ERRATUM

Article on Effect of Cortical Extract on Glucose Tolerance of Adrenalectomized and Hypophysectomized Rats, by Howard A. Ball, Leo T. Samuels and H. F. Schott, 1937, **35**, 633, the authors wish to acknowledge the financial aid of the Committee on Scientific Research of the American Medical Association.

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SECTION MEETINGS

IOWA	
State University of Iowa	February 25, 1937
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Effect of Cholecystectomy on Extra-Hepatic Ducts in the Rabbit and Guinea Pig.

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It is well known that removal of the gall bladder in man and dog is usually followed, in time, by dilation of the extra-hepatic ducts. In these species the output of bile by the liver is comparatively small, the ability of the gall bladder to concentrate hepatic bile is large, so that the gall bladder can accommodate the 12- to 24-hour output of the liver in fasting (*i. e.*, the "physiologic capacity" of the gall bladder is great). Further, the sphincteric resistance is high and the ducts are not motile. In the guinea pig and rabbit the output of bile by the liver is relatively very large and the ability of the gall bladder to concentrate hepatic bile is small, so that their gall bladder can accommodate only one-fiftieth or less of the 24-hour output of hepatic bile. Further, their sphincteric resistance is relatively small and their common duct is motile, a sort of an extra-duodenal ampulla, which is contractile, being present in the guinea pig (Table I).

TABLE I.
Effect of Cholecystectomy on Dog, Man, Rabbit, and Guinea Pig.

Species	24-hour bile output per kilo	24-hr. bile output per gm. of liver	Physiological capacity* of gall bladder	Sphincteric resistance in mm. N saline	Common duct measurements in mm. (dia)	
					Normal	After cholecystectomy
Dog	17.0	0.51	12+	300-700	2.70	5- 7.0
Man	16.0	0.64	12+	300-700	6-7.0	10-15.0
Rabbit	118.7	4.53	0.6	35-70	3.02	4.0
Guinea pig	228.0	6.50	0.3	40-50	1.92	1.57

* Hours of hepatic secretion accommodated by gall bladder.

It was thought that the extra-hepatic ducts of the rabbit and guinea pig might not dilate after cholecystectomy, it being conceived that the factors of low sphincteric resistance and of motility of the common duct might operate to prevent or definitely reduce dilation. In fact, Burget and Brocklehurst[1] have reported that only slight dilation occurred in 2 of 7 guinea pigs from 24 to 33 days after cholecystectomy. We have repeated and extended their experiments.

A standardized method was adopted to measure accurately the diameter of the common bile duct in normal and cholecystectomized animals. A small glass cannula connected to a manometer (5 mm. bore) was inserted into the distal end of the common duct which was then filled with normal salt solution under pressure of a column of fluid 10 cm. in height. The diameter of the distended duct was then measured with a micrometer graduated to 0.01 mm.

The gall bladder and the immediately adjacent portion of the cystic duct was removed under ether anesthesia, trauma to the common duct being avoided. The animals showed no ill effects from the operation and were maintained thereafter on a stock diet.

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[1] Burget and Brocklehurst, *Am. J. Physiol.*, 1928, **83**, 578.

Results. Rabbits. Ten rabbits were cholecystectomized, the postoperative period lasting from 9 to 200 days. The average diameter of the common duct in 5 normal rabbits (average weight 1952 grams) was 3.02 mm. as compared to 4.00 mm. in the cholecystectomized group (average weight 2011 grams). It will be noted in Table II that the diameter of the common duct was within normal limits in 4 of the 10 cholecystectomized rabbits.

Guinea Pigs. The diameter of the common duct averaged 1.92 mm. in 6 normal guinea pigs (average weight 543 gm.) and 1.57 mm. in 6 guinea pigs 18 to 137 days after cholecystectomy (average weight 441 gm.) (Table II).

TABLE II.
Common Duct Measurement in Normal and Cholecystectomized Rabbits and Guinea Pigs.

Normal			Cholecystectomized			
No.	Wt. in gm.	Diameter common duct in mm.	No.	Wt. in gm.	Days without gall bladder	Diameter common duct in mm.
Rabbits						
1	1500	2.75	1	2000	9	3.05
2	3000	3.25	2	1400	30	4.00
3	1620	3.30	3	1600	53	4.10
4	2050	2.80	4	950	128	3.50
5	2050	3.00	5	1670	128	4.03
			6	1530	129	4.58
			7	2400	142	5.60
			8	2956	148	3.07
			9	3650	180	4.92
			10	1956	200	3.05
Aver.	1952	3.02	Aver.	2011		4.00

$$\frac{\text{Body weight}}{\text{Common duct diameter}}\ \text{ratio} = \begin{matrix}\text{Normal } 0.001547\\ \text{Cholecystectomized } 0.001988\end{matrix}$$

Normal			Cholecystectomized			
No.	Wt. in gm.	Diameter common duct in mm.	No.	Wt. in gm.	Days without gall bladder	Diameter common duct in mm.
Guinea pigs						
1	860	2.02	1	360	18	1.50
2	810	2.50	2	380	18	1.00
3	525	2.25	3	410	46	1.60
4	365	1.45	4	500	137	1.50
5	360	1.72	5	600	137	2.07
6	338	1.58	6	395	137	1.72
Aver.	543	1.92	Aver.	441		1.57

$$\frac{\text{Body weight}}{\text{Common duct diameter}}\ \text{ratio} = \begin{matrix}\text{Normal } 0.00354\\ \text{Cholecystectomized } 0.00356\end{matrix}$$

Burget and Brocklehurst did not provide measurements of the common duct after cholecystectomy in the guinea pig and stated that they doubted the significance of the slight dilation they observed. Our observations show that dilation does not occur in the guinea pig; if so it is slight and occurs rarely. In the rabbit, however, some dilation does occur, the average percent increase in diameter of the common duct being less than 30% (body weight taken into consideration). In view of the facts that the common duct of the rabbit is relatively less motile than that of the guinea pig and that the sphincteric resistance is about the same in both, the absence of dilation in the guinea pig is best accounted for by the greater motility of its duct. Thus, in the guinea pig the gall bladder is unimportant either as a reservoir for storage of bile or as a pressure regulatory mechanism. In the rabbit it apparently serves to a slight extent as a pressure regulatory mechanism.

A study of the comparative anatomy of the gall bladder, to be reported elsewhere, shows that the gall bladder is a variable anatomic character in rodents. Some, like the mouse, have apparently retained the organ for purposes of pressure regulation and storage, as in primates and carnivours; others, like the rat, have completely lost the organ; and others, like the guinea pig, have retained the organ. In the latter, however, it can be of no real physiologic significance, since anatomic changes do not result in the extra-hepatic ducts after cholecystectomy.

Conclusions. Cholecystectomy in the guinea pig results in no dilation of the common bile duct. Cholecystectomy in the rabbit results in a slight but variable dilation of the common duct.

9128 P

On the Relation Between Hypoglycemia and Anoxemia.

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Studies on the O_2 uptake by excised brain tissue and the O_2 consumption of the brain in man indicate that the utilization of oxygen by the brain is dependent on the sugar concentration in the blood. Brain tissue obtained from insulinized animals shows a lesser oxygen consumption than controls (Holmes[1]) and the arterio-venous oxygen difference is diminished after the administration of insulin in man (Dameshek and Myerson[2]). The anoxemia theory of insulin convulsions is further strengthened by experiments of Glickman,[3] who found, in this laboratory, that the interval between the injection of insulin and the occurrence of convulsions in rats was considerably shortened if the rats were exposed to a lower barometric pressure which in itself did not have any material effects on rats under control conditions. It was attempted to clarify the relationship between anoxemia and hypoglycemia by blood pressure studies in which the blood pressure response of the anesthetized dog to oxygen deficiency was determined at various blood sugar levels. The experiments were carried out on 18 dogs narcotized with sodium barbital or sodium amytal. Air with 6.2% oxygen was inhaled from Douglas bags for 3 minutes. This concentration produced ordinarily either no rise in blood pressure or only a very slight one. If, however, insulin was administered the blood pressure response was augmented considerably and the rise increased with decreasing blood sugar concentration as is indicated in the examples given in Table I. The phenomenon is reversible, as is shown by the fact that the injection of 30% glucose reduced the blood pressure response approximately to normality corresponding to the elevation of the blood sugar. The typical reaction was found to be independent of blood pressure changes which may have occurred in the experiment. It will be the objective of further studies to determine whether other sugars than glucose are able to restore the normality the blood pressure response to oxygen deficiency and whether any changes in the concentrations of ions, etc., in the blood, which may

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[1] Holmes, E. G., *Biochem. J.*, 1930, **24**, 914.

[2] Dameshek, W., and Myerson, A., *Arch. Neurol. Psychiat.*, 1935, **33**, 1.

TABLE I.

Effect of Inhalation of 6.2% Oxygen on Blood Pressure in Dogs.

		Effect of 6.2% O_2 on blood pressure in mm. Hg.		
Time	Blood sugar mg. %	Before 6.2% O_2	Max. rise During 6.2% O_2	Remarks
12:03 P.M.	90	95	95	10 kilo dog injected with .60 gm. sod. amytal intraperitoneally 8:30 A. M. 11:39—72 units of insulin injected intravenously.
12:55 ’’	60	98	148	
1:25 ’’	45	96	160	
12:25 ’’	90	135	146	12 kilo dog injected with .72 gm. sod. amytal, 9:00 A.M. 80 units of insulin injected intravenously 12:47 P.M. At 3:47 P.M. 7 cc. glucose given intravenously. (30%.)
2:44 ’’	76	110	145	
3:28 ’’	70	112	158	
3:50 ’’	86	110	138	

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[3] Glickman, N., *Proc. Am. Physiol. Soc.*, 1936, p. 61.

occur after administration of large doses of insulin, have any causal relationship to the phenomenon observed.

Conclusion. The rise in blood pressure occurring during the inhalation of 6.2% oxygen for 3 minutes increases in proportion to the fall of blood sugar obtained after intravenous injection of large doses of insulin in dogs. Intravenous injection of glucose which offsets to a certain extent the hypoglycemia induced by insulin diminishes the blood pressure response to oxygen deficiency.

9129 P

Production of Small-Colony Variants of *Staphylococcus aureus.*

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We have previously reported the production of small-colony variants, or G forms, after prolonged serial transfer of *S. aureus* in lithium chloride broth or plain broth media.[1, 2] Swingle[3] subsequently confirmed these results and also obtained these forms after extended growth in nutrient broth or casein-digest medium. Under these conditions the variants occur only rarely and in small numbers, seldom exceeding 1.0% of the colonies on a plate. Chinn[4] in a study of the metabolic activity of the small colony variants of *S. paradysenteriæ* Sonne and *S. aureus* found them to be metabolically far less active than the normal forms, having a reduced generation-time, reduced fermentative powers, lessened ability to reduce methylene blue and a slower cataphoretic velocity. This lowered metabolic activity and the ability to revert to forms identical with the parent culture furnishes some evidence that small-colony variants may be forms whose metabolism has been greatly reduced by some unknown factor.

Quastel and Woolridge[5] studying the enzymes of *B. coli* found that among many inorganic salts barium chloride was the most effective in inhibiting the metabolic activity of washed suspensions of these organisms. These results, and the known lowered metabolic

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[1] Hoffstadt, R. E., and Youmans, G. P., *J. Infect. Dis.*, 1932, **51**, 216.

[2] Hoffstadt, R. E., and Youmans, G. P., *J. Bact.*, 1934, **27**, 551.

[3] Swingle, E. L., *J. Bact.*, 1935, **29**, 467.

[4] Chinn, B. D., *J. Infect. Dis.*, 1936, **59**, 137.

[5] Quastel, J. H., and Woolridge, W. R., *Biochem. J.*, 1927, **21**, 1224.

activity of the small-colony variants, indicated that barium chloride might be effective as an incitant of their production.

Concentrations of barium chloride of 0.5 to 5.0% were made up accurately in 1.0% peptone solution. Since *S. aureus* produces only slight growth in plain peptone medium the acetone-soluble portion of meat extract containing Hughes'[6] growth factor was added. This was prepared by extracting 50 gm. of meat extract dissolved in 100 cc. distilled water with 6 to 8 volumes of acetone. The acetone was decanted and removed *in vacuo* at 50 to 60°C. The residue was taken up in 50 cc. of distilled water and re-extracted with 6 to 8 volumes of acetone which was decanted from the precipitate and removed as before. The final residue was dissolved in 50 cc. distilled water and after the pH had been adjusted to 7.0 was sterilized by filtration through a Berkefeld N filter. Four percent of this activating solution was added to the barium chloride peptone medium after the latter had been adjusted to pH 7.0 and sterilized either by Berkefeld filtration or by autoclaving. This final medium was tubed aseptically in 5 cc. amounts. All media were tested for sterility before use.

Fifteen strains of *S. aureus* were employed. After a preliminary 24-hours' growth in peptone-activator medium a constant amount was inoculated with a capillary pipette into media containing varying concentrations of barium chloride and into the same medium which did not contain barium chloride. These cultures were incubated at 37°C. and subcultures upon meat infusion agar plates made every 2 days. The plates were examined for the presence of small-colony variants after 24 to 48 hours' incubation.

The ability of the different strains of *S. aureus* to grow upon the barium chloride medium varied considerably. The completely inhibiting concentrations of barium chloride varied from 1.0 to 5.0% with different strains, though the ability of any one strain to grow in certain concentrations was relatively constant. Growth, as judged by both turbidity and plating, was very slow, sometimes requiring from 2 to 4 days to become evident. This was especially true in the higher concentrations of barium chloride.

The small-colony variants appeared very rapidly under the influence of barium chloride, all strains showing their presence within 6 days and most of them within 2 days. (Table I.) Four strains were completely changed to the small-colony form after 4 days' incubation and 7 of the strains after 6 days. These results are in marked contrast to those obtained by older methods which ordinarily

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[6] Hughes, T. P., *J. Bact.*, 1936, **23**, 437.

TABLE I.

Approximate percentage of small colony variants found upon plates made from Barium Chloride Broth Cultures of *Staphylococcus aureus.*

Time in days	Strains of *Staphylococcus aureus*														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
2	10	100	10	90	50	NG*	NG	90	50	75	NG	NG	NG	NG	NG
4	10	100	10	95	25	75	75	90	95	99	100	NG	NG	100	100
6	50	100	25	100	25	10	75	100	95	100	50	100	50	100	100

* No growth.

require weeks or months to produce less than 1.0% of small-colony variants. By the use of barium chloride complete conversion into the variant form may take place within 4 days. The number of small-colony variants produced was roughly proportional to the concentration of barium chloride in which growth was obtained, the higher concentrations producing a relatively higher percentage of variants. Control cultures of the broth containing no barium chloride did not produce small-colony variants even after 30 days of incubation. The chlorides of sodium, magnesium and calcium were without effect.

The variants obtained in the above manner differed in no way from those obtained by other methods. The colonies on agar were extremely small and cultures exhibited the same lowered metabolism, the same manner of reversion and the same ability, if properly handled, to maintain their variant-characteristics over periods of weeks or months without reversion.

When barium chloride was added to plain meat infusion or meat extract broth the appearance of the small-colony variants was very irregular. Upon the barium chloride peptone-activator medium, however, consistent results were always obtained.

The rapidity of appearance and the great number of small-colony variants produced in the presence of barium chloride tends to support the theory that these variants of *S. aureus* are merely forms possessing a lowered rate of metabolism which have evolved under the influence of an unfavorable environment. Furthermore, the work of Quastel and Woolridge[5] showing the effectiveness of barium chloride as an inhibitor of the enzymatic functions of *B. coli* offers a possible explanation of the mechanism by which barium chloride brings about the production of these small-colony variants.

9130 P

Precipitin Reactions of Various Ovalbumins.

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Chicken, pearl guinea fowl and amherst pheasant ovalbumins* were crystallized 4 times by the method previously described for chicken ovalbumin by Cole.[1] These crystallized proteins, together with a purified chicken-blood albumin, were injected into rabbits and the antiserums produced were tested with the various antigens by the precipitin-reaction. Chicken-blood albumin was included in these experiments because we had previously shown that it is immunologically identical with the conalbumin in chicken egg-white,[2] so that an antiserum against this protein may be used to detect the presence or absence of conalbumin in supposedly pure preparations of the other proteins in egg-white. The results obtained are summarized in Tables I and II. The figures represent the highest dilu-

TABLE I.
Precipitin Reactions of Various Ovalbumins.

Antigens	Serums Against			
	Chicken Ovalbumin (1957)	Chicken Conalbumin (1958)	Pearl Guinea Fowl Ovalbumin (1963)	Amherst Pheasant Ovalbumin (1965)
Chicken Ovalbumin	100,000	0	10,000	10,000
Chicken Conalbumin	0	100,000	0	0
Pearl Guinea Fowl Ovalbumin	0	0	10,000	100,000
Mother Liquor, P. G. F. Ovalbumin	0	10,000	10,000	10,000
Amherst Pheasant Ovalbumin	0	0	10,000	100,000
Mother Liquor, Amherst Pheasant Ovalbumin	0	1,000,000	1,000,000	100,000

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* We are indebted to Mr. Rudyerd Boulton, of the Field Museum of Natural History, for the amherst pheasant and pearl guinea fowl eggs used in this investigation.

[1] Cole, A. G., PROC. SOC. EXP. BIOL. AND MED., 1933, **30**, 1162.

[2] Hektoen, L., and Cole, A. G., *J. Infect. Dis.*, 1928, **42**, 1.

TABLE II.
Absorption Experiments with Various Ovalbumins.

Antigens	Chicken Ovalbumin Antiserum	Pearl Guinea Fowl Ovalbumin Antiserum		Amherst Pheasant Ovalbumin Antiserum	
	+ Chicken Ovalbumin	+ Chicken Ovalbumin	+ P. G. F. Ovalbumin	+ Chicken Ovalbumin	+ Amherst Pheasant Ovalbumin
Chicken Ovalbumin	0	0	0	0	0
Pearl Guinea Fowl Ovalbumin	0	100,000	0	100,000	0
Amherst Pheasant Ovalbumin	0	10,000	0	10,000	10,000

For these experiments, 0.5 cc. antiserum was mixed with 0.1 cc. of a 1% solution of the antigen indicated. After standing for one hour at room temperature, the precipitate formed was centrifuged off and the undiluted supernatant liquid was used for precipitin tests with fresh portions of each antigen.

tion of the antigen which, when layered over some of the undiluted antiserum, still gave a visible precipitate at the interface between antigen and antiserum in one hour at room-temperature.

As indicated in Table I, the antiserum against chicken ovalbumin is specific, reacting only with the homologous antigen. On the other hand, the antiserums against the pearl guinea fowl and amherst pheasant ovalbumins react not only with the homologous antigens, but also with the other 2 crystallized ovalbumins. None of these crystallized proteins react with conalbumin-antiserum, while their mother-liquors, which contain some of the crystallizable as well as the non-crystallizable albumin in the egg-white, give strong reactions with this antiserum. These reactions of the mother-liquors with an antiserum against chicken conalbumin indicate that the conalbumins in these various egg-whites are either similar or identical, and, judging from the results of our earlier work on chicken-blood albumin and conalbumin, that the blood-albumins of these fowl are also similar or identical. The complete absence of cross-reactions between the crystallized ovalbumins and the conalbumin indicates that each of the ovalbumins used in these experiments is a pure substance, entirely free from contamination with the conalbumin which appears to be a common constituent of all these egg-whites. We are thus led to the conclusion that whereas chicken ovalbumin gives rise to a single, specific antibody, the injection of pearl guinea fowl or amherst pheasant ovalbumin leads either to the formation of several distinct antibodies, or else a single antibody capable of reacting with the various ovalbumins. In an attempt to choose between these two possibilities, absorption-experiments were carried out with the various antigens and antiserums. The results, summarized in Table II, indicate that chicken ovalbumin removes its own, specific precipitin from all 3 antiserums, and has no effect on any other precipitins which may be present in the serums. On the other hand, the pearl guinea fowl ovalbumin removed all of the precipitins from the pearl guinea fowl antiserum, while the amherst pheasant ovalbumin, in the amount added to the amherst pheasant antiserum, removed all heterologous precipitins but not the homologous one. In these cases, therefore, it appears that the injection of an apparently pure, homogeneous antigen has led to the formation of at least 2, and possibly more, distinct precipitins. These results are in accord with those of Hooker and Boyd[3] on chicken and duck ovalbumins and of Landsteiner[4] on azoproteins. This work is being extended to include a number of other ovalbumins.

9131

Chondroitin in Canine Anaphylaxis.

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The accidental discovery of Crandall and Roberts[1] that the administration of chondroitin sulphuric acid to patients with migraine frequently results in marked relief has not been explained. Since both the physiological action of chondroitin and the pathogenesis of migraine are only imperfectly understood, the therapy is of course empirical. Migraine has, however, been considered by a number of investigators as an allergic manifestation. Also chondroitin has been shown to have a protective action against liver injury due to a variety of causes.[2] The relationship between the liver and the anaphylactic reaction in the dog is well known. Consequently it was hoped that a study of the effect of chondroitin administration upon the anaphylactic reaction in the dog might contribute some information to these various and possibly related problems.

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[3] Hooker, S. B., and Boyd, W. C., *J. Immunol.*, 1936, **30**, 41.

[4] Landsteiner, K., The Specificity of Serological Reactions, Springfield, Charles C. Thomas, 1936.

[1] Crandall, L. A., and Roberts, G. M., *Ill. Med. J.*, 1933, **63**, 513.

[2] Crandall, L. A., Roberts, G. M., and Snorf, L. D., *Am. J. Dig. Dis. and Nutr.*, 1936, **3**, 289.

Chondroitin was administered to 13 horse-serum sensitized dogs in doses of 10 gm. per day. In 8 animals it was given during the last 10 days of the incubation period, and in 5 it was given for 10 days prior to the sensitizing injection and throughout the incubation period. The animals were anesthetized so that the degree of shock resulting from the assaulting dose of serum could be recorded by the blood pressure tracing. Definite shock occurred in all animals, 3 reactions being fatal, the remainder moderate to severe. The distribution of the various grades of severity of shock was identical with that in a large number of controls.[3] Consequently it is concluded that the administration of chondroitin does not influence in any way the anaphylactic reaction in the dog.

9132

Purified Bacteriophage from Lysogenic Cultures.

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The desirability of freeing bacteriophage from extraneous materials, such as media constituents, bacterial proteins and products of bacterial growth, has long been recognized and needs no emphasis. Efforts to obtain phage in a pure state have been numerous, and reports concerning the properties of phages of relative purity indicate that they differ from broth phage. The purpose of this paper is to describe a simple method for obtaining phage of low nitrogen content without the use of special media or equipment.

Bordet and Ciuca[1] noted that the late bacterial growth occurring after the period of maximum phage action continued to carry the phage through a series of transfers on artificial media. They called these lysogenic* cultures. During the course of a study of cultures having this property, it was observed that the growth from agar slants could be washed repeatedly with physiological salt solution or distilled water, and that even the twenty-seventh washings still showed the presence of phage in easily detectable amounts. It seemed reasonable to suppose that the earlier washings would remove the greater part of other proteins and that later washings might contain the phage in purer form; it was found, in fact, that repeated washings of lysogenic cultures could be used as a means of purifying phage for *B. coli, Staphylococcus aureus,* and *B. graveolens.* The phage for *B. coli* was chosen for further investigative work because of its relative stability.

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[3] Mills, M. A., and Dragstedt, C. A., *J. Immunol.*, 1936, **31**, 1.

[1] Bordet, J., and Ciuca, M., *Compt. rend. de Soc. de Biol.*, 1920, **83**, 1293.

* The term 'lysogenic' was later applied to and is now commonly used for cultures of the type first described by Lisbonne and Carrère;[2] *i. e.*, recently isolated cultures (usually *B. coli*) whose filtrates contained phage active on heterologous strains (usually dysentery bacilli of the Shiga or Flexner type).

[2] Lisbonne, M., and Carrère, L., *Compt. rend. de Soc. de Biol.*, 1922, **86**, 569.

Bacteriophage active in the 10^{-8} dilution was prepared from sewage for a non-sucrose fermenting strain of *B. coli* recovered from human feces. A 1:10 dilution in broth was incubated with the susceptible bacteria for 3 to 5 days. Lysis occurred, followed by an overgrowth from which a lysogenic culture was prepared by transferring 2 loopfuls to a beef extract agar slant. Scattered colonies appearing in 24 hours were streaked over the surface of the slant and the growth resulting in 3 to 5 days was suspended in 2 or 3 cc. of sterile distilled water. Six-inch petri dishes with a thin layer of 2% agar were inoculated with 2 drops of this suspension, which were then streaked over the surface of the plates with sterile bent glass rods. After 48 hours' incubation, the growth was washed from the surface of the plates into physiological salt solution or distilled water, using 3 cc. per plate. The suspension was centrifuged three times in 50 cc. round-bottomed centrifuge tubes. The supernatant fluid was carefully decanted each time, the sediment drained and resuspended in approximately the same amount of fluid. The first, second and third washings were filtered through Berkefeld N or W filters, new filters being used for the third supernatant fluid to prevent the addition of extraneous nitrogen by the procedure. Titrations for phage content were made by the serial dilution method in broth, separate pipettes being used for each dilution; also 3 plaque counts were made on different dilutions of each phage sample.

Although the titer sometimes dropped slightly in successive washings, the titer of the third supernatant fluid was consistently high (10^{-7}) (see Table I). The total nitrogen content of the third washings, determined by the microkjeldahl method of Koch and McMeekin,[3] ranged from 0.66 to 0.88 mg. per 100 cc. in different experiments, varying slightly with the unavoidable slight variation in the number of bacteria washed and the amount of suspending

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[3] Koch, F. C., and McMeekin, T. L., *J. Am. Chem. Soc.*, 1924, **46**, 2066.

TABLE I.
Phage titer and total nitrogen content of representative samples of phage washed from lysogenic cultures.

Experiment	Supernatant fluid	Total nitrogen mg./100 cc.	Phage titer
Growth washed from agar plates into physiological salt solution.			
A	1	74.92	10^{-8}
	2	3.34	10^{-7}
	3	0.66	10^{-7}
B	3	0.88	10^{-7}
C	3	0.84	10^{-7}
Growth scraped from agar plates into distilled water.			
D	1	6.06	10^{-7}
	2	0.62	10^{-7}
	3	0.31	10^{-7}
E	1	5.82	10^{-7}
	2	1.01	10^{-7}
	3	0.27	10^{-7}
Crude broth phage		180.0	10^{-8}

fluid used each time. Rabbits immunized with the third supernatant fluid, containing 0.36 to 0.49 mg. of nitrogen in the amount of phage injected, produced no agglutinins for the homologous organism detectable in a 1:10 dilution of serum and only faintly demonstrable precipitins with antigen undiluted or diluted 1:2 (none with antigen diluted 1:4). Antibodies for bacteriophage were present in dilutions from 1:320 to 1:640.

Because of the faint precipitin reaction, indicating the presence of some bacterial protein, the technique of making the phage was modified slightly. The 48-hour growth of lysogenic cultures on agar plates was scraped with a rubber kitchen plate scraper instead of being washed from the surface of the medium. The suspension was then washed in sterile distilled water as previously described. The reduction in total nitrogen content occurring as a result of this modification (from between 0.66 and 0.88 mg. to between 0.27 and 0.31 mg. per 100 cc.) indicated that a large part of the nitrogen in previous samples had come from medium proteins. The possible sources of the remaining nitrogen are the filters, the glassware, the water (distilled only once), the bacteria and the phage.

Phage purified in the manner described containing 0.27 to 0.31 mg. of nitrogen per 100 cc. was concentrated from 25 cc. to approximately 0.5 cc. by filtering through collodion membranes having an average pore diameter of 34 millimicrons prepared by the method of Bauer and Hughes.[4] The titer was increased 5 to 7 times by this procedure. It is believed that this may also serve as a means of further purification.

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[4] Bauer, J. H., and Hughes, T. P., *J. Gen. Physiol.*, 1934, **18**, 143.

Complete inactivation of the purified phage occurred in 30 minutes by exposure to 65°C., whereas homologous broth phage of similar titer (10^{-7}) and pH (6.1 to 6.2 by the indicator method) required a temperature of 75°. The purified phage was completely inactivated by 50% C. P. acetone in 24 hours at room temperature, though broth phage of similar titer and pH still had a titer of 10^{-5} after the same period of time under the same conditions. After evaporation to dryness in a Freas vacuum oven at 22°C. and resuspension in one-fourth the original volume of water, the titer of the purified phage was 10^{-2}; that of the crude broth phage, 10^{-6}. These results appear to support the findings of other workers that purified phage is more susceptible to inactivation by physical and chemical agents than is crude broth phage.

Summary. Serological tests and nitrogen determinations indicate that bacteriophage prepared by repeated washing of lysogenic cultures of *B. coli* contains little bacterial or other protein. Methods of preparation and concentration of such purified phage are described and some of its properties compared with those of homologous crude broth phage of similar titer and pH.†

9133

Comparative Study of Water Metabolism in Amphibians Injected with Pituitrin.

F. R. STEGGERDA. (Introduced by W. C. Rose.)

From the Department of Physiology, University of Illinois, Urbana.

Since the work of Brunn[1] it has been shown by several other investigators that frogs *(Rana pipiens)* when injected with pituitrin, increase markedly in weight due to the absorption of water. This increase, occurring in the case of frogs, gave rise to the problem of comparing the amount of water uptake in different animals in the class Amphibia when similarly treated.

The animals selected for this comparative study were: the toad *(Bufo americanus)*, mud puppy *(Necturus maculatus)*, and two species of frogs *(Rana pipiens* and *Rana clamitans)*. This selection affords a wide variation in natural environmental conditions, the toad living in dry surroundings and the mud puppy constantly in water; as to the 2 species of frogs used, the pipiens reside primarily in damp, marshy fields some distance from the streams, while the clamitans are found more or less submerged in pools of water along the stream's edge. The experiments were carried out in the late summer and early fall.

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† The collodion membranes used in this work were prepared by Dr. Evelyn B. Tilden of the Department of Research Bacteriology.

[1] Brunn, F., *Z. ges. exp. Med.*, 1921, **25**, 170.

The procedure was similar to that used in previous experiments.[2] The animals were nearly submerged in tap water at room temperature for at least 12 to 18 hours before the experiment. They were then removed from the water separately, dried with gauze as uniformly as possible, and weighed accurately to one-tenth of a gram. They were next injected subcutaneously with obstetrical pituitrin (Parke Davis), one-tenth cc. per 10 gm. of body weight (one international unit), and again returned to the water. Routine weigh-

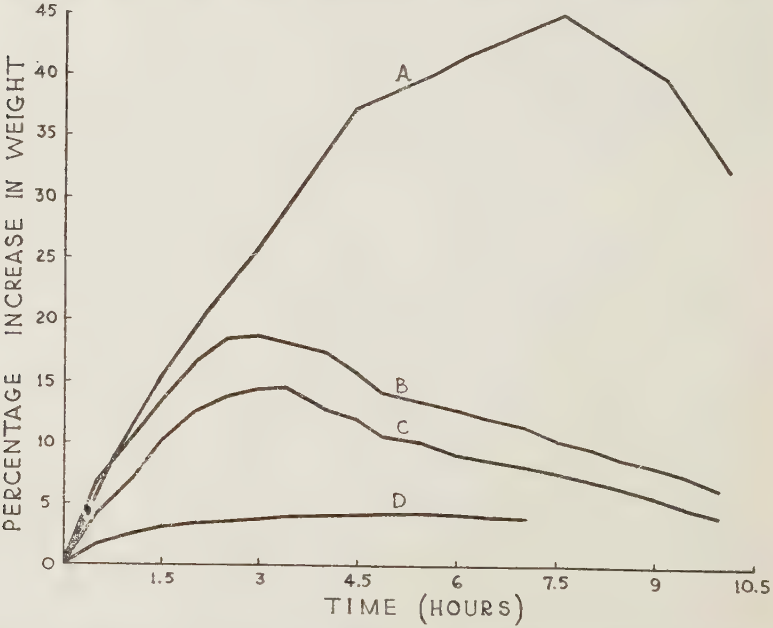

FIG. 1.

Each of the above curves represents the average percentage weight changes due to water absorption in 6 animals.

A. Toad *(Bufo americanus)*.
B. Frog *(Rana pipiens)*.
C. Frog *(Rana clamitans)*.
D. Mud Puppy *(Necturus maculatus)*.

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[2] Steggerda, F. R., and Essex, H. E., PROC. SOC. EXP. BIOL. AND MED., 1934, **32**, 425.

ings were made at one-half to one hour intervals for 7 to 10 hours. Any changes in weight were calculated on the basis of percentage variation from normal. In all cases the experiments were controlled with uninjected animals kept in the same environment and weighed at the same time intervals. These control animals never showed any appreciable changes in weight and therefore will not be referred to further.

The results of these experiments (see curve) show that the injection of pituitrin into the toad caused a very marked uptake of water with an average increase in weight of nearly 45% in 7½ hours, after which there was a gradual return to normal. On the other hand, the mud puppy, whose natural environment is water, showed an increase of only 4%. The *Rana pipiens* gave an increase of about 18% in weight in 3 hours, which is in agreement with our previous findings; the average increase in the clamitans, with the same dose of pituitrin, was consistently about 4% less than that of the pipiens. These observations indicate that the response of the animal to pituitrin may be related to its natural habitat.

Since the above results show so great a difference in the uptake of water following the injection of pituitrin, a limited number of experiments were carried out to find whether there was also a similar difference in the animals studied regarding the amount of water they could lose by dehydration before death. Six each of the *Rana clamitans* and *pipiens* and 4 toads were weighed and placed in separate cages in the breeze of an electric fan for 2½ hours. During this time the average percentage weight lost in the 2 species of frogs was approximately 22%, while that of the toads was 36%. The mud puppy was not studied in these experiments because they survive so short a time after being removed from water.

After this dehydration period the animals were placed in water to record their ability to recover. Four of the 6 clamitans died, whereas all the pipiens and toads recovered their lost weight and survived. This shows an interesting correlation with the previous experiments regarding the ability of animals to withstand environmental changes.

Regarding the exact nature of the comparative response of these animals to hydration or dehydration, little can be said at the present time. Earlier experiments[3] on frogs with cloaca tied would lead one to deduce that the hydration effect was due primarily to the increased skin permeability. Other experiments[4] indicate that the skin changes are not definitely related to a melanophore dilation which accompanies pituitrin injections. Recently, however, Rey[5] reported that the hydrating response in frogs to pituitrin is not alone due to the increased absorption through the skin, but that the decreased kidney output and an increased water retention by certain tissues play an important rôle in causing the increase in body weight. The work of Novelli[6] with skin pouches in which the circulation was left intact indicates that the skin permeability is in some way affected by pituitrin to allow an increase transfer of water.

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[3] Steggerda, F. R., *Am. J. Physiol.*, 1931, **98**, 255.

[4] Oldham, F. K., *Am. J. Physiol.*, 1936, **115**, 275.

Although it is admitted that the exact nature of the transfer of water in amphibians after the injection of pituitrin is still obscure, it can be concluded from the above experiments that there is a definite correlation between the amount of water interchange and the natural habitat of the animal.*

9134

An Ensemble for Determination of Circulation Time of Blood By an Ionization (Geiger Chamber) Method.

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A decade ago there appeared the first of a series of publications by Blumgart and his associates[1] concerning the use of injections of the disintegration products of radon (radium emanation) for the determination of the time elapsing between the introduction of the radioactive material at a given point in the blood-vascular system and its arrival at some other point in the system as detected by a shielded ionization chamber.

In the early investigations the radioactive material was injected into the cubital vein of one arm and detection of its arrival in the radial artery of the other arm was accomplished by means of a modification of the cloud-chamber devised by Wilson.[2] In the later studies Blumgart and his coworkers used ionization chambers of the type designed by Geiger[3] and a mechanism for recording the time of passage of the active material between the chosen points. The ensemble of apparatus was somewhat cumbersome, unwieldy and expensive. A compact, portable and relatively inexpensive type of equipment has been assembled and used by us in investigations concerning the circulation time of the blood between 2 points (in general, between the left jugular vein and the right femoral artery, lying in the adductor canal) and as an adjunct in the series of investigations concerning blood flow in dogs by Herrick, Essex, Mann and Baldes.[4] The apparatus and method have the advantages of absence of trauma, utilization in trained animals, deletion of reaction times and of immediate and positive detection of the injected radioactive material. By reason of the desire expressed by various investigators for information concerning the experimental ensemble and procedure, we are presenting the essential features.

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[5] Rey, P., *Comp. rend. Soc. de biol.*, 1935, **118**, 949.

[6] Novelli, A., *Rev. Soc. Argent. Biol.*, 1936, **12**, 163.

* These experiments were begun at the University of Michigan Biology Station at Douglas Lake.

* Fellow in Biophysics, The Mayo Foundation. Now associated with the Department of Physics, Iowa State College, Ames, Iowa.

[1] Blumgart, H. L., and Yens, O. C., *J. Clin. Invest.*, 1927, **4**, 1. Blumgart, H. L., and Weiss, Soma, *J. Clin. Invest.*, 1927, **4**, 389.

The ensemble of apparatus for the detection and recording of the radioactive material at the time of its arrival at the chosen point in the blood-vascular system consists essentially of 4 parts: an ionization (Geiger) chamber and attached insulated cable, a supply of power of high voltage for the ionization chamber, three stages of amplification with power supplies, and a mechanism for recording the receipt of ionizing energy or changes in ionization in the Geiger chamber. The unit is operated from a 110 volt, 60 cycle, source of alternating current through a voltage stabilizer; the recording circuit requires, in addition, a supply of direct current of about 120 volts.

The wiring diagram of the amplifying system and power supplies is too complicated to be suitable for reproduction. This portion of the apparatus was assembled for us by the General Radio Company, Cambridge, Mass., under the designation of Type GR-P-361 Geiger Counter; diagrams and details of construction and operation may be obtained from them or from one of the authors (C.S.) of this communication.

The ionization chamber is attached to a flexible cable which, in turn, is so constructed as to prevent leakage and to obviate the discharges caused by changes in capacity due to movements of the cable. The cable is 8 feet in length in order that the ionization chamber may be conveniently and advantageously placed in any desired location during the investigations of the time of circulation of the blood. The ionization chamber (Fig. 1), designed for ease

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[2] Wilson, C. T. R., *Proc. Roy. Soc. Med.*, Series A, 1911, **85**, 285.

[3] Geiger, Hans, and Rutherford, E., *Phil. Mag.*, 1912, **24**, 618. Geiger, Hans, *Phys. Z.*, 1913, **14**, 1129.

[4] Herrick, J. F., Essex, H. E., Mann, F. C., and Baldes, E. J., *Am. J. Physiol.*, 1934, **108**, 621.

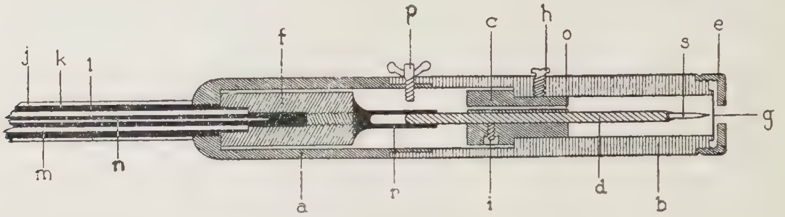


FIG. 1.
Details of construction of the ionization chamber and attached cable. The significance of the letters is indicated in the text.

of replacement of the needle point (s) or aluminum window (g), is made of a cylinder of brass. The window of aluminum foil (0.0125 mm. thick) is protected by a screw cap (e) containing an aperture 3 mm. in diameter; the needle (a polished phonographic needle with a tempered point) is fastened into a brass rod (d) mounted in the axis of the cylinder. The two portions (a, b) of the chamber may be separated by removing the wing nut (p). The rod carrying the needles is held in its retaining bushing (c) of hard rubber by means of a set screw (i). The point of the needle is placed about 1 mm. from the aluminum foil.

The center of the *flexible cable* consists of a 7 mm. high-tension cable (l, n), the core (n) of which is soldered to the holder (r) of the rod carrying the needle. Rubber tubing (m), of 7.5 mm. internal diameter, is forced over the central cable. Flexible copper shielding (k) is stretched tightly over the rubber tubing and soldered to the ionization chamber. An outer layer of machinists' tape completes the construction of the cable. These details regarding the construction of the ionization chamber and cable have been given because of the difficulties encountered in eliminating fairly frequent and relatively prolonged discharges from the needle point (charged positively and just under the break-down potential of about 2500 v.) to the window of the ionization chamber. Following the plan of construction as outlined, we found that a sharp, decisive blow to the cable was necessary to change the capacitance enough to cause momentary discharges in the chamber.

The *output* from the amplifying system is impressed on a *recording system* which is shown in diagrammatic form in Fig. 2. The relay (y) acts as a vibrator switch; its period is longer than the time required for the deionization of the thyratron tube (t) or of the ionization chamber. Activation of the relay motivates the recording point (p). Records of the to and fro movements of the point (p) which occur during the receipt of ionizing energy in the Geiger chamber are made on a strip of paper moving with a constant known velocity. For reasons which will be obvious from the data to be presented in other communications regarding the circulation time of the blood as influenced by various physico-physiological agents and by drugs, we found it desirable to employ gears giving, respectively, 1, 2, 4 and 8 cm. of travel of the paper in one second. In Fig. 2, S_3 indicates the insertion of the leads from a switch, of spring brass, attached to the plunger of the syringe used for purposes of injection; when this switch is closed, a record of the commencement and the duration of time of injection of radioactive material is made on the moving strip of paper.

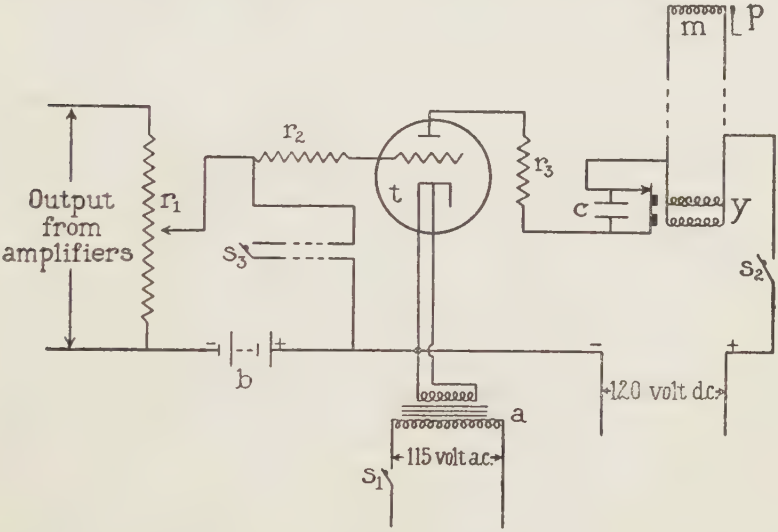
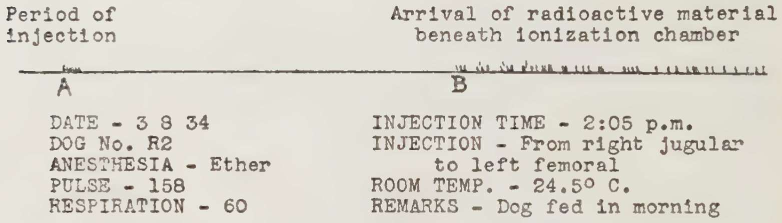


FIG. 2.
Diagram of recording circuit. The significance of the letters is indicated in the text.

The *radioactive material* which is used as the ionizing agent and which is injected into the animals consists of a solution of the disintegration products of radium emanation (radon). One of these active products, radium C, has a marked emission of gamma rays. Since gamma rays are highly penetrating and active ionizing agents, they serve as the principal source for the production of discharges in the Geiger chamber. The transformation of radioactive products proceeds very rapidly to radium D. In about 2 hours the activity of the injected material has decayed sufficiently to allow of the use of the same animal in other tests. The injection of a solution of the radioactive material obtained from 3 to 5 millicuries of radon gives satisfactory records of the circulation time.

Satisfactory *shielding of the ionization chamber* is accompanied by the use of cubical lead blocks about 7.5 cm. on a side. These blocks are mounted on suitable rods and supports and so arranged that 2 of them, in the shape of a V, are placed as close to the body of the animal as possible. The ionization chamber is placed in the crotch formed by the lead blocks. Additional screening is secured by lead plates 0.5 cm. thick, 10 cm. wide and 25 cm. long, placed over the abdomen and just in front of the lead blocks, respectively. This procedure reduces the number of extraneous discharges to an occasional one only.



DATE - 3 8 34
DOG No. R2
ANESTHESIA - Ether
PULSE - 158
RESPIRATION - 60

INJECTION TIME - 2:05 p.m.
INJECTION - From right jugular to left femoral
ROOM TEMP. - 24.5° C.
REMARKS - Dog fed in morning

FIG. 3.

Reproduction of a photograph of an original record (reduced about a third). The circulation time of the blood in this case was 5.5 seconds. The distance A to B represents the time between injection of radioactive material in the jugular vein and its arrival in the femoral artery.

Fig. 3 is the reproduction of a record (reduced about one-third) obtained for use in determining the time of circulation of the blood between the right jugular vein and the right superficial femoral artery of a dog under light ether anesthesia. The circulation time of the blood between these two points is determined from the known velocity of the recording paper and the distance between the commencement of injection and the first indication of the arrival of the radioactive material beneath the window of the ionization chamber.[5]

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[5] Sheard, Charles, McCracken, E. C., and Essex, H. E., *Am. J. Physiol.*, 1934, **109**, 96.

9135 P

Correlation between Ocular Stimulation and Spermatogenesis in the English Sparrow (*Passer domesticus*).*

A. R. Ringoen and A. Kirschbaum. (Introduced by R. F. Blount.)

From the Departments of Zoology and Anatomy, University of Minnesota.

The importance of daily light ration in the sexual cycle of certain birds is well established. Male English sparrows may be brought to full spermatogenic activity within 6 to 8 weeks during the fall and early winter by daily exposures of 6 to 7 hours (4 P. M. to 11 P. M.) to the light afforded by either a 60 or 100 watt incandescent bulb.[1-4] No significant gonadal response, however, is observed in female birds during a comparable period of exposure. Female response is considered in a subsequent publication.

The question has been raised as to the manner in which light brings about testicular hypertrophy in the bird. Ivanova[5] placed caps over the eyes of male sparrows exposed to increased daily light ration to determine the rôle of the eyes in the response of the testes to light. According to her interpretation, light rays exert their stimulating effect on the testis largely through the general body surface as the breeding season approaches (February and March). Benoit[6, 7] performed similar experiments with ducks and concluded that light acts through the ocular region. The stimulating action of light was maintained even after the optic nerves were severed. Bissonnette[8] was unsuccessful in an attempt to ascertain whether added exposures of light affect capped starlings since the birds died as the result of handling.

Thirty-five juvenile male sparrows were used in the present study. Fifteen of these received 7 hours of additional light daily beginning November 5th. A second group of 9 birds was capped during the 7-hour light exposure. Nine caged birds and 2 specimens from nature

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*Aided by a grant from the research funds of the Graduate School of the University of Minnesota.

[1] Kirschbaum, A., *Anat. Rec.*, (Suppl.), 1933, **57**, 62.

[2] Witschi, E., *Wilson Bull.*, 1935, **47**, 177.

[3] Kirschbaum, A., and Ringoen, A. R., *Anat. Rec.*, 1936, **64**, 453.

[4] Riley, G. M., Proc. Soc. Exp. Biol. and Med., 1936, **34**, 331.

[5] Ivanova, S., *Arch. Exp. Path. u. Pharmakol.*, 1935, **179**, 349.

[6] Benoit, J., *C. R. Soc. Rend.*, 1935, **118**, 669.

[7] Benoit, J., *Anat. Rec.* (Suppl.), 1936, **67**, 81.

[8] Bissonnette, T. H., *Quart. Rev. Biol.*, 1936, **2**, 371.

TABLE I.

Testis length, diameter, volume and degree of spermatogenic activity of four groups of juvenile sparrows. "0" is indicative of spermatogonia; "+" spermatocytes; "++" spermatids and occasionally sperms; "+++" complete spermatogenic activity.

No. of Bird	Killed Dec.	Days of Added Light	Bill Color	Body Weight gm.	Left Testis			Germ Cells
					Length mm.	Diam. mm.	Volume mm.^{3}	
				Cap Birds				
1	16	41	Black	28	5.0	4.0	42.0	+
2	16	41	Yellow	25	3.3	3.0	15.6	+
3	16	41	"	24	1.4	1.2	1.1	0
4	17	42	"	28	2.0	1.4	2.1	0
5	19	44	"	26	2.1	1.4	2.2	0
6	19	44	"	29.5	1.3	1.1	.8	0
7	19	44	"	27.5	1.4	1.1	.9	0
8	19	44	"	29	2.8	2.1	6.5	+
9	19	44	"	29	1.9	1.0	1.0	0
Aver.		42.8		27.5	2.4	1.8	8.0	
Temp.—57°-74°F.								
				Light Birds				
1	13	38	Black	26	7.0	7.0	180.1	+++
2	16	41	"	31	6.1	4.0	51.2	++
3	16	41	"	30.5	5.2	4.0	43.6	+++
4	17	42	"	30.5	10.0	7.2	272.1	+++
5	17	42	Yellow	30.5	2.0	1.5	2.4	0
6	19	44	Black	29	7.4	5.2	131.2	+++
7	19	44	"	31	7.0	4.5	74.0	+++
8	19	44	"	28	7.3	6.2	146.8	+++
9	19	44	Yellow	24.5	1.3	1.0	0.7	0
10	19	44	Black	27	7.6	6.8	184.0	+++
11	19	44	"	28	8.0	6.2	160.8	+++
12	21	46	Yellow	23	3.2	2.4	9.6	+
13	21	46	Gray	28	5.0	3.8	37.6	++
14	21	46	Yellow	22	4.6	3.2	24.8	+
15	22	47	Black	29	8.3	6.0	156.4	+++
Aver.		43.5		28.0	6.0	4.6	98.4	
Temp.—57°-74°F.								
				Caged Controls				
1	16	0	Yellow	34	1.5	1.2	1.1	0
2	16	0	"	25.5	1.2	1.1	.8	0
3	16	0	"	29	1.5	1.2	1.1	0
4	17	0	"	27	1.3	1.0	.7	0
5	21	0	"	25.5	1.4	1.0	.7	0
6	21	0	"	29.5	1.5	1.2	1.1	0
7	21	0	"	30	1.7	1.1	1.1	0
8	22	0	"	30	2.0	1.7	3.0	0
9	22	0	"	27.5	1.7	1.3	1.5	0
Aver.		0		28.5	1.5	1.2	1.2	
Temp.—56°-70°F.								
				Outside Controls				
1	21	0	Yellow	25	2.0	1.3	1.8	0
2	22	0	"	28	1.5	1.2	1.1	0
Aver.		0		26.5	1.8	1.3	1.5	

served as controls. All caged sparrows were allowed to feed 8 hours daily; the temperature was approximately the same for all these specimens. The experiment extended over a period of 6 weeks; results are shown in Table I.

Sections of the testes from the "light birds" revealed numerous spermatozoa in 9 of the 15 specimens. The gonads of 2 L and 13 L contained only occasional sperms. Slight spermatogenic activity was evident in 12 L and 14 L. Lack of response was noted in 5 L and 9 L. These results confirm those previously reported by the authors.[3]†

The outcome of the experimental studies on the capping of male sparrows is shown in the table. Failure of definite gonadal response is strikingly evident in 6 of the 9 capped birds subjected to additional light. The gonads of the other 3 contained spermatocytes. All control testes were spermatogenically inactive.

Our results indicate that testicular response to light in the sparrow (if experiments are begun in early November) depend largely on reception of the stimulus through the ocular region.‡

9136

Arachidonic and Linolic Acid of the Serum in Normal and Eczematous Human Subjects.*

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Relatively little information is available concerning the content and nature of the various unsaturated fatty acids of the blood. The

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† Although Riley recognizes the influence of light in promoting spermatogenic activity experimentally in the quiescent sparrow testis, he concludes that an "intrinsic sexual rhythm" independent of light influence is operative.

‡ According to Whetham, E. O., (*J. Agric. Sci.*, 1933, **23**, 383), "There would seem to be two possibilities concerning the action of light on the reproductive organs by way of the anterior pituitary. Firstly, it may be a quantitative effect on some precursor substance similar to that by which ultra-violet light forms vitamin D; or secondly it may cause stimulation of the anterior pituitary by acting on sensory nerves." Quoting further from Whetham's publication, "Although the rays at the red end of the spectrum have greater penetrating power on animal tissues and so might act on the tissues generally, it would seem more probable in view of the facts at present available that the stimulus acts by way of the nerves associated with color vision."

* Aided by grants from the Medical Research Fund of the Graduate School of the University of Minnesota and the National Live Stock and Meat Board.

work of Hansen[1] and Faber and Roberts[2] which showed that the fat of the serum tends to be less unsaturated in infants with outspoken eczema than in normal healthy infants of similar ages, indicates the necessity of a more detailed study of the constituent fatty acids of the serum. The studies of Burr and Burr[3] and Brown and Burr[4] which point to the essential nature of linolic acid in animal nutrition, naturally bring up the question of the possible significance of this particular fatty acid in relation to the nutrition and metabolism of the human subject. Also, because of its high degree of unsaturation, relatively slight shifts in the content of arachidonic acid of the serum could substantially alter the iodine number of the blood fat. While no specific function of arachidonic acid is known it has been suggested that it plays an important rôle in the intermediary metabolism of fat.[5] The present report deals with the quantitative determination of the insoluble bromides of these particular fatty acids in the sera of normal subjects and patients with eczema.

After aliquots of the lipid extract of the serum were taken for the determination of the cholesterol, the total fatty acids and the iodine absorption capacity of the serum, the remaining portions were pooled in the following classes: a—normal subjects; b—children with eczema; c—eczema cases in clinical remission. Individual samples contained the equivalent of 1 to 3 cc. serum and were obtained from a variable number of subjects, the age groups of which were comparable with the one exception of the group of older patients with active eczema. The extracts of the total lipids were kept in air tight containers in the dark and saved until there was the equivalent of 15 to 50 cc. of serum present. Two sets of samples were determined for each class mentioned. The following technique for the determination of arachidonic and linolic acids was used:

The solvent was first evaporated from the serum extract under vacuum at a temperature below 65°C. and the lipid residue immediately weighed. The necessary amount of alcoholic KOH was added and the lipids were saponified for 30 minutes on a steam bath. After saponification, the soaps were quickly cooled, extracted with ether to remove the unsaponifiable material, and then acidified with dilute HCl to free the fatty acids.

The fatty acids were removed from the acidified solution by 3 washings with peroxide-free ether and the ether solution containing the fatty acids then washed free from the HCl with distilled water, using a separatory funnel. The bulked aqueous washings were finally extracted with ether to recover the traces of fatty acids carried into the aqueous phase and this solution again washed free of HCl with water before being added to the fatty acid sample. The amount of fatty acid loss with this technique was found to be negligible.

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[1] Hansen, Arild E., Proc. Soc. Exp. Biol. and Med., 1933, **30**, 1198.

[2] Faber, H. K., and Roberts, D. B., *J. Pediat.*, 1935, **6**, 490.

[3] Burr, Geo. O., and Burr, M. M., *J. Biol. Chem.*, 1929, **82**, 345; 1930, **86**, 587.

[4] Brown, Wm. R., and Burr, Geo. O., Presented at Am. Chem. Soc., Section of Biol. Chem., Kansas City, Missouri, April 16, 1936.

[5] Wesson, L. G., *J. Biol. Chem.*, 1925, **65**, 235.

All traces of ether and water were removed at 65°C. under vacuum, and the dried fatty acids then taken up in petroleum ether, transferred to 15 cc. centrifuge tubes and the insoluble material centrifuged out of solution. The petroleum ether soluble substances were then transferred to a 25 cc. Erlenmeyer flask, the solvent completely removed under vacuum and weighed.

The weighed fatty acids were next dissolved in about 5 cc. anhydrous ether, chilled below 0°C. and brominated with dry bromine gas. After 12 hours' storage at —18°C. the brominated acids were returned to a centrifuge tube, care being taken to remove all traces of the precipitated bromides from the Erlenmeyer flask by the use of additional anhydrous ether. The excess wash ether was removed from the tubes by evaporation with an electric fan.

When the ether solution had been reduced to approximately 5 cc., the precipitate was centrifuged out. This polybromide precipitate was washed until snow white, using one cc. of anhydrous ether for each washing. Usually 3 or 4 washings were required for this purpose. The polybromides were then completely freed of solvent, weighed and calculated as arachidonic acid, the substance indicated by the bromine content of the precipitate, as determined by the Willard-Thompson method.[6]

The ether solution containing the remainder of the brominated fatty acids was then titrated with dilute sodium sulphite to remove the free bromine and the solution evaporated off by an electric fan. When the flask contents were completely water-free (usually about 48 hours), they were extracted with anhydrous ether. The ether solution was centrifuged to remove suspended material, transferred to a second centrifuge tube, and the ether then evaporated off. Five cc. petroleum ether was added to the contents of the centrifuge tube and the mixture stirred, stoppered, and stored for at least 12 hours in a refrigerator. Upon removal from the refrigerator, the precipitate of tetrabrome-linolic acid was centrifuged from solution and washed with petroleum ether, the same technique as described for the polybromides being used.

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[6] Willard, H. H., and Thompson, J. J., *J. Am. Chem. Soc.*, 1930, **52**, 1893.

In these precipitations it was found that if the amount of solvents used was reduced to the minimum requirement to carry the precipitate, the loss due to the precipitate solubility was unweighable with a high-grade analytical balance.

Results. The results are summarized in Table I and are expressed in percentage of the total fatty acid present.

TABLE I.
Content of Linolic and Arachidonic Acids in Pooled Samples of the Sera from Normal and Eczematous Subjects.

Type	Arachidonic Acid (Polybromides) % of Total Fatty Acid	Linolic Acid (Tetrabromides) % of Total Fatty Acid
Normal		
35 subjects	2.83	4.80
12 ”	2.90	5.20
Eczema (active)		
18 subjects (younger group)	1.34	3.20
8 ” (older group)	1.60	4.20
Eczema (treated—in remission)		
12 subjects	1.40	4.80
5 ”	0.76	5.40

As regards the two samples of blood from the normal subjects, there is a striking agreement in the content of the arachidonic and linolic acid, and the quantity of these acids present in these samples is very similar to that found in a large sample of blood from a normal adult human being.[7] The arachidonic acid value is comparable to that reported by Tängl[8] for ox blood serum. In both samples of serum from the eczematous subjects the content of both linolic and arachidonic acids was definitely diminished. This finding confirms the previously reported observations[1, 2] that the total fatty acids of the serum from children with eczema have a lower iodine number than do those of normal children of comparable ages, also, that the difference in iodine number is not as marked in older children with eczema as in the younger age group.[9] The possible significance of these findings in relation to factors such as age, diet and infection, which are known to affect the degree of unsaturation of the serum lipids, is discussed in a current publication.[9]

The specimens from the patients with eczema in remission were obtained from those who were in good condition clinically, most of them having been treated by the administration of raw linseed oil or corn oil internally for variable lengths. However, part of the sample was obtained from patients who had been treated with local application of ointments, containing crude coal tar. The values of linolic acid were definitely higher in these treated groups, being in the same range as found for normal subjects. It seems only reasonable to believe that this increase in the linolic acid content is explained by the fact that diets of these subjects for the most part contained fats which were rich in linolic acid. This finding, however, does not prove that the return to normal values of the linolic acid content is concerned with changes in the clinical picture noted in these cases. There was no effect on the arachidonic acid content of these specimens, although in one instance the values were definitely lower. Our conjecture is that linolic acid (or at least the unsaturated fatty acids), which may be necessary for normal skin nutrition, is probably one of the many factors disturbed in eczema.

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[7] Brown, Wm. R., Hansen, Arild E., Burr, G. O., and McQuarrie, Irvine, PROC. SOC. EXP. BIOL. AND MED., in press.

[8] Tängl, H., *Biochem. Z.*, 1930, **226**, 180.

[9] Hansen, Arild E., *Am. J. Dis. Child.*, 1937, in press.

Summary and Conclusions. Linolic acid (tetrabromides calculated as such) is present in human serum to the extent of about 5% of the total fatty acids, and arachidonic acid (polybromides calculated as such) about 3% of the total fatty acids in pooled samples of blood serum from normal children. The content of both these fatty acids is definitely diminished in children with eczema. In those cases of active eczema which were clinically cured either by the internal administration of oils rich in the unsaturated fatty acids or by the local application of ointments containing crude coal tar, or both, the arachidonic acid was not increased while the linolic content was the same as that found in normal subjects. These findings indicate a possible reason for the low iodine number of the serum fatty acids in eczema and further suggest that a disturbance in the metabolism of the unsaturated fatty acids may be one of the many factors at fault in this condition.

9137

Determination of Staphylococcal Types by Fermentation of Mannite.*

L. A. JULIANELLE.

From the Oscar Johnson Institute, Washington University School of Medicine, St. Louis.

The recent demonstration that different strains of staphylococci elaborate chemically distinct carbohydrates[1] has made it possible to

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* Conducted under a grant from the Commonwealth Fund of New York.

[1] Julianelle, L. A., and Wieghard, C. W., PROC. SOC. EXP. BIOL. AND MED., 1934, **31**, 947; also, *J. Exp. Med.*, 1935, **62**, 23.

separate these organisms into immunological types.[2, 3] Differentiation of the types, however, is dependent upon precipitation of the specific polysaccharides in homologous antiserum, a technic requiring the extraction of the reactive substances. Since this procedure is somewhat cumbersome, necessitating time and facilities not always available, it seems desirable to introduce a simpler and more rapid means for the determination of types. Consequently, several methods have been studied for this purpose, and while none possesses an absolute accuracy, the reaction of fermentation of mannite appears to offer the best possibilities from the aspect of simplicity, rapidity and precision. It is proposed, therefore, to report in this communication the correlation of staphylococcal types to fermentation of mannite.[4]

The data bearing on this study, arranged in the form of a protocol, are presented in Table I. It will be seen that an analysis has been made of 102 strains of staphylococci, which were tested in sequence as they were isolated in the diagnostic laboratory of Barnes Hospital. In 47 strains, the type was determined by precipitation of carbohydrates, while in 55, the staphylococci were separated presumptively into Type A or B depending chiefly on their derivations.

TABLE I.
The Relation of Staphylococcal Types to Fermentation of Mannite.

Source	No. of strains	Pigment		Type		Reaction in mannite	
		Albus	Aureus	A	B	+	—
	(a) Type determined by precipitation-test						
Pathogenic	31	4	27	31	0	30	1
Non-pathogenic	16	16	0	0	16	1	15
	(b) Type determined presumptively						
Pathogenic	31	2	29	31	0	30	1
Non-pathogenic	24	24	0	0	24	2	22

In deciding upon pathogenicity of the given strains, complete reliance was placed on source, since attempts to differentiate the different organisms by animal-inoculation revealed frequent discrepancies between virulence for man and animal, thus indicating a species-variation difficult of reconciliation. Since the figures for both determined and presumptive types are closely comparable, it seems fair in summarizing to consider them as one. Thus, then, of 62 pathogenic strains, all Type A, 6 produced white pigment, and 56, varying de-

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[2] Julianelle, L. A., and Wieghard, C. W., *J. Exp. Med.*, 1935, **62**, 11.

[3] Thompson, R., and Khorazo, D., *J. Bact.*, 1937, **33**, 51.

[4] Julianelle, L. A., *J. Bact.*, 1937, **33**, 49.

grees of yellow pigment. Two strains were unable to ferment mannite after a period of incubation of one week, and 60 caused fermentation in intervals of less than 24 hours to 3 and 5 days. In many instances, prolonged incubation following fermentation was accompanied by an alkalinization sufficient to overcome the acidity originally formed. Of the 40 non-pathogenic strains, all Type B, and all *albus,* only 3 fermented mannite. It is interesting to note that while a slight variation from yellow pigment occurred among the Type A strains, all of the non-pathogenic strains of Type B observed remained constant in elaborating only white pigment.

The data reveal, therefore, that of a total of 102 cultures of staphylococci studied, it is possible to differentiate the immunological types A and B by fermentation of mannite within an error of 5%. This is in close agreement with the results observed by both Thompson and Khorazo[3] and S. T. Cowan of England.[5]

9138 P

Formation of Glucose-1-Phosphoric Acid in Muscle Extract.

G. T. CORI AND C. F. CORI.

From the Department of Pharmacology, Washington University School of Medicine, St. Louis.

In recent experiments[1] on minced and washed frog muscle, adenylic acid, in amounts corresponding to 4 mg. P per 100 gm. muscle, increased very markedly the formation of hexosemonophosphate. The small amount of phosphorylation which occurred without addition of adenylic acid was attributed to the presence of nucleotides which had not been removed by washing, since the washed muscle contained 2 to 4 mg. % of organic P.

The first phosphorylation product was found to be glucose-l-phosphoric acid which was isolated as the crystalline brucine salt and which has since been synthesized and identified as an α-compound. This is of significance in view of the fact that glycogen, the carbohydrate from which this ester is formed, consists of α-glucosidic linkages.

Glucose-l-phosphoric acid, when added to fresh muscle extract, is converted in a few minutes to hexose-6-phosphoric acid by a wandering of the phosphate group. The same change occurs in minced and washed muscle but at a slower rate, so that after short periods of incubation and with phosphorylation accelerated by the addition of adenylic acid, the rate of formation of the 1-ester exceeds its rate of conversion to the 6-ester, these being the conditions which made possible the isolation of the new ester.

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[5] Personal communication.

[1] Cori, C. F., and Cori, G. T., PROC. SOC. EXP. BIOL. AND MED., 1936, **34**, 702.

TABLE I.

Rabbit muscle extract dialyzed for 17 hours (0.01 mg. of organic P per cc.). 22 cc. extract + 12 cc. M/3 phosphate buffer pH 7 + 165 mg. glycogen + 65 mg. $MgCl_2.6H_2O$. Incubated at 24°. All determinations carried out in hexosemonophosphate fraction (= water-soluble barium salts, precipitated with alcohol). Values in mg. per 10 cc. of extract-buffer mixture.

Time min.	Without adenylic acid					With adenylic acid (0.36 mg. P per 10 cc. mixture)					
	1-ester†		6-ester*			1-ester†			6-ester*		
	hexose	P found	hexose	P found	P calc.	hexose	P found	P calc.	hexose	P found	P calc.
30	0.2	0.04	4.0	0.74	0.69	8.3	1.42	1.43	7.2	1.36	1.24
60	0.4	0.05	5.8	1.01	1.0	4.6	1.02	0.79	18.9	3.12	3.26
90	0.1	0.0	7.4	1.25	1.28	1.2	0.15	0.21	22.4	3.92	3.86

* Hexose-6-phosphoric acid, calculated from reducing power and organic P content before hydrolysis.

† Glucose-1-phosphoric acid calculated from increase in reducing power and inorganic P after 10 min. hydrolysis in N H_2SO_4.

The experiments in Table I show that a dialyzed rabbit muscle extract behaves in the same way as minced and washed frog muscle. Without addition of adenylic acid phosphorylation was slow and glucose-1-phosphoric acid did not accumulate in significant amounts. With adenylic acid added phosphorylation was increased about 4 times, resulting in an accumulation of glucose-1-phosphoric acid. Between 60 and 90 minutes phosphorylation no longer occurred and the gain in 6-ester (+ 3.5 mg.) was balanced by the decrease in 1-ester (— 3.4 mg.).

Further experiments showed that such small amounts of adenylic or inosinic acid accelerate phosphorylation that, had they been originally present in the dialyzed muscle extract, they could not have been detected by analytical methods. The smallest amount added corresponded to 0.1 mg. of adenylic acid P per 100 cc. of original extract. This amount still increased phosphorylation 3.5 times over that occurring in the extract without added adenylic acid, corresponding to a transfer of about 140 molecules of inorganic phosphate per molecule of added adenylic acid. Inosinic acid added in equivalent amounts was about one-half as active as adenylic acid. The assumption seems justified that the muscle extract used in these experiments (and other extracts similarly prepared), since it was able to phosphorylize and convert the 1- to the 6-ester, contained minute amounts of nucleotides and of magnesium which were not removed by dialysis.

Magnesium has been shown to be an essential part of the coenzyme system of muscle.[2] In the experiments in Table II the total amount of phosphate esterified was the same without and with addition of magnesium, but the conversion of the 1- to 6-ester was much more rapid when magnesium was added. If magnesium is needed for phosphorylation, much smaller amounts are necessary than are required for the conversion of the 1- to 6-ester. When synthetic α-glucose-1-phosphoric acid was added to a dialyzed extract, addition of magnesium accelerated very markedly the transformation of this ester to the 6-ester (which was isolated as the barium salt and identified as Embden ester). At pH 5.9 in the presence of magnesium the wandering of the phosphate group was practically abolished, but the total amount of phosphate esterified was not significantly less than at pH 7.2. Heating the extract for 10 minutes at 60° caused an inactivation of the enzyme responsible for the wandering of the phosphate group.

TABLE II.

Effect of magnesium on transformation of the 1- to the 6-ester. Experimental conditions were the same as in Table I, except as noted. Values in mg. per 10 cc. extract-buffer mixture.

Adenylic (A) or inosinic (I) acid added. mg. per 10 cc. mixture	Magnesium added mg. per 10 cc. mixture	Period of incubation	1-ester	6-ester	Total esterification
			as hexose		
A .007	0	30	8.1	1.6	9.7
A .007	2	30	0.7	7.6	8.3
A .15	0	60	12.8	4.0	16.8
A .15	2	60	1.0	15.5	16.5
I .15	0	60	5.3	3.8	9.1
I .15	2	60	0.1	9.0	9.1
0	0	60	1.4	2.7	4.1
0	2	60	0.0	3.9	3.9

Adenylic acid has been shown to act as acceptor for phosphate in organic combination, namely for the phosphate split off from phosphocreatine,[3] phosphopyruvic acid[4] or hexosediphosphoric acid.[5] The present experiments as well as those previously reported[1] indicate that adenylic acid can mediate still another reaction, namely, the transfer of inorganic phosphate in the formation of hexosemonophosphoric acid from glycogen. Inosinic acid is also, but less active.[6] In a dialyzed muscle extract to which adenylic or inosinic acid has been added as well as in a dialyzed extract incubated without addition of nucleotide, the first phosphorylation product is glucose-l-phosphoric acid. Addition of magnesium ions is without effect on phosphorylation but accelerates markedly the conversion of glucose-1- to hexose-6-phosphoric acid.

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[2] Lohmann, C., *Biochem. Z.*, 1931, **237**, 445.

[3] Lohmann, C., *Biochem. Z.*, 1934, **271**, 264.

[4] Ostern, P., Baranowski, T., and Reis, J., *Biochem. Z.*, 1935, **279**, 85.

[5] Lutwak-Mann, C., and Mann, T., *Biochem. Z.*, 1935, **281**, 140.

9139

Transmission of Estrogenic Substances from Animal to Animal.

ISABELLA H. PERRY AND ALLAN PALMER. (Introduced by Professor C. L. Connor.)

From the Departments of Pathology and of Obstetrics and Gynecology, University of California Medical School, San Francisco.

We have been treating female white mice with massive doses of estrone (200 I.U.) painted on the skin.* To our astonishment a group of 25 untreated spayed mice, whose vaginal smears were all negative, all went into estrus. It had been our custom in treating or examining animals to take them from their cage, treat, and then place in a temporary box until all animals in a cage were removed, then return them to their cage. No transmission of estrogenic substances has been noted among a large number of female mice treated with subcutaneous injections of 1-10 I.U. of estrone.

To trace out the source of estrogenic stimulation we placed a standard mouse in the cage of each group receiving estrone, and one in the temporary box during a morning when the mice were being treated with estrone. A standard mouse is a young spayed adult known to respond to 2 I.U. of estrone administered subcutaneously. All of these standard mice went into estrus and remained in estrus for about 2 weeks. There could be only 2 probable routes of transmission and of reception of the estrogenic substance, *i. e.,* from the skin, and from the excreta, and through the skin, and through the mouth. To make a crude estimate of these factors we placed a standard mouse with a muzzle on in the cage with estrone-treated animals. A mouse in a paraffin sack, with only its head exposed, was also placed in a cage with estrone-treated animals. Two other muzzled and sacked animals were placed in the bottoms of cages of estrone-treated animals. The cages have false bottoms. Except for the period of exposure each standard mouse was caged separately. All four of these mice went into persistent estrus, indicating that all four probable routes are effective.

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[6] Parnas, J. K., and Mochnacka, I., *Compt. rend. Soc. Biol.,* 1936, **123**, 1173.

* We are grateful to Dr. Oliver Kamm of Parke, Davis and Company for the preparation of "Theelin" used in these experiments.

The volume and precision of work on the sex hormones, and on carcinogenic substances will increase and, in some instances, overlap. The transmission of estrogenic substances from animal to animal has not been previously reported. This observation is recorded in the hope that it will prevent such confusion as occurred concerning vitamin B, due to the transmission of the vitamin from animal to animal by excreta. The significance of the transmission of estrogenic substances from animal to animal is: one, that where animals are being treated with large amounts of estrogenic substances they should be kept segregated from any other animals under observation for estrus; and, two, where precision in dosage is required, animals should be kept in individual cages with false bottoms.

9140

Method of Preparing Mice for Quantitative Determination of Urinary Estrogen.*

ALLAN PALMER. (Introduced by C. D. Leake.)

From the Department of Obstetrics and Gynecology, University of California Medical School.

The methods of biological assay for urinary estrogen in use at the present time are dependent upon the production of estrus in a castrated mouse or rat. Various, modifications of the original Allen-Doisy test have been used, such variations being single or multiple injections of either aqueous, glycerol, or oily extracts into castrated or immature normal mice or rats. Methods utilizing male animals and fish have not as yet proved of any great value for quantitative purposes.

Chemical methods for the determination of urinary estrogen have

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* Supported in part by the Christine Breon Fund for Medical Research.

been proposed by Kober,[1] Cohen and Marrian,[2] David,[3] Cartland *et al.*,[4] Schmulovitz and Wylie,[5, 6] and Pincus *et al.*[7] Chemical tests based on color changes in the test tube and comparison with standards are preferable to biological tests. The disadvantage of chemical tests is chiefly their inapplicability to the estimation of estrogens in the urine of non-pregnant women. For this purpose we must, for the present, still resort to biological means.

A biological test for urinary estrogen must be for the biological activity of all the estrogenic substances present in the urinary extract. The test cannot be specific for, or differentiate between, different forms of the hormone. The advantage of the biological method is its ability to demonstrate very small amounts of estrogenic hormone in the presence of inactive impurities. The disadvantages are the troublesome time consuming procedures and the necessity of maintaining a supply of test animals. The greatest disadvantage is the source of error and variation in methods. More than a 4000% variation can be demonstrated in the procedures[8] now used.

This paper is presented in an effort to decrease the large percent of error and variation. Our test animal by choice has been the castrated adult female mouse. Just as chemically pure hormone for assay is tested against a known standard hormone, so should urinary extracts for quantitative hormone determination be tested against a standard, or in animals that have been prepared with a known standard hormone preparation.

When daily vaginal smears from large groups of adult virgin mice were examined over a period of two to three weeks, from 30-40% of these animals were found to be having regularly occurring estrus cycles. The animals in such a group were castrated and tested with an estrogen, and it was found that those animals having a cycle reacted more consistently to the minimal effective dose of estrone than those not having a cycle. The method employed at first was to follow a group of animals from day to day from the standpoint of their vaginal smears and castrate them as soon as estrus occurred. Almost without exception these animals, so castrated, responded one week later in full estrus to the minimal dose of estrone which we found to be 0.1 gamma. Later it was found to be more time-saving to castrate a group of animals and one week later inject all of them with 0.1 gamma of estrone. It was found that the same percentage (30-40%) of animals responded to the minimal dose and we had excluded the necessity of taking smears beforehand. In a separate experiment it was found that as little as 0.05 gamma estrone would produce estrus, but this was rare and inconstant as compared with the reactivity to 0.1 gamma. If an active animal were castrated and allowed to remain unstimulated for too long a time (10-14 days or longer) it could not be expected to, and will not respond to the minimal or even several times the minimal dose of estrone. Fortunately 7 days has proven to be a good interval for the artificial production of estrus in a castrated animal by the injection of known or unknown amounts of estrogen.

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[1] Kober, S., *Biochem. Z.*, 1931, **239**, 209.

[2] Cohen, S. L., and Marrian, G. F., *Biochem. J.*, 1934, **28**, 1603.

[3] David, K., *Acta brev. Neerland.*, 1934, **4**, 64.

[4] Cartland, G. F., Meyer, R. K., Miller, L. C., and Rutz, M. H., *J. Biol. Chem.*, 1935, **109**, 213.

[5] Schmulovitz, M. J., and Wylie, H. B., *J. Lab. and Clin. Med.*, 1935, **21**, 210.

[6] Schmulovitz, M. J., and Wylie, H. B., *J. Biol. Chem.*, 1936, **116**, 415.

[7] Pincus, G., Wheeler, G., Young, G., and Zahl, P. A., *J. Biol. Chem.*, 1936, **116**, 253.

[8] MacCorquodale, D. W., Thayer, S. A., and Doisy, E. A., *J. Biol. Chem.*, 1936, **115**, 435.

After the animal has been so prepared and tested it is ready for use as a group I animal in testing unknown extracts. Injections of extract are made weekly and the vaginal reaction is followed from day to day. No animal is used for testing an extract oftener than once in 2 weeks. The second week after testing an extract the animal is always given a standard dose whether it has responded to the previous test dose or not. The animals will not always respond to this standard dose again and if not the dose is repeated the following week. *No animal is used for testing again that has not responded in full estrus to a known standard dose one week before.*

The next and most recent step forward has been a plan whereby more than 30-40% of the animals castrated can be made use of in our determinations. We were able to make use of three groups of animals. Group I consisted of those responding to 0.1 gamma, group II those responding to 0.2 gamma, and group IV, those responding to 0.4 gamma estrone. Experimental details are omitted, but the plan evolved is as follows: Each of a group of animals is castrated and one week later given 2.0 gamma estrone in 0.2 cc. olive oil subcutaneously. All the animals react to this overdose, obviating the necessity of taking smears during the second week. On Monday of the third week each animal is injected with 0.4 gamma of estrone. Those responding are given 0.2 gamma the fourth week. Those not responding to 0.4 gamma are discarded. Those responding to 0.4 gamma and not to 0.2 are grouped as IV. Those responding to 0.2 are given 0.1 gamma. Those responding to 0.2 and not to 0.1 gamma are grouped as II, while those responding to 0.1 gamma are grouped as I. In following the three groups of animals from day to day, one must take into account the same principle of keeping each animal controlled as described above for group I. The group I animals can be used for testing a short while only and soon have to be put into the II group. The latter eventually comes to be the largest as it draws from groups of newly castrated animals as well as from group I. The usefulness of the animals in group IV is also transitory, some of them becoming II's and the others shortly becoming inactive to 0.4 gamma estrone.

The problem of proper variation in the spacing of the amount of urinary extract to be injected presented itself. Interpolated values must be as close together as possible for the sake of accuracy and yet far enough apart so that the inherent variability of the individual animal would not present itself to the extent that two animals receiving a given dose of urinary extract would not react at all, while one or both of the two animals receiving the next smaller dose would go into full estrus. We resorted to trial and error methods and the amounts shown in the accompanying table seem most practical. Two animals are injected with each dose. The test is made for no less than 1.0 gamma per liter of urine since we have never found less in the urine of normal adult women. Extracts are 100-fold concentrates of the urine. Their preparation is by benzene extraction, combining the method of Cohen and Marrian[9] for maximal recovery with minimal destruction of hormone and a suggestion from Schmulovitz and Wylie[10] which makes use of the protective and washing properties of sodium carbonate.

TABLE I.
Injection Table.

Urinary extract (whole)			Urinary extract (1-10 Dil.)			Urinary extract (1-100 Dil.)		
Test dose cc.	*Group*	*Value* γ	*Test dose* cc.	*Group*	*Value* γ	*Test dose* cc.	*Group*	*Value* γ
1.0	I	1.0/L	0.5	II	40.0/L	0.5	II	400.0/L
0.5	I	2.0/L	0.3	II	66.6/L	0.3	II	666.6/L
0.5	II	4.0/L	0.2	II	100.0/L	0.2	II	1000.0/L
0.3	II	6.6/L	0.1	II	200.0/L	0.1	II	2000.0/L
0.2	II	10.0/L	0.1	IV	400.0/L	0.1	IV	4000.0/L
0.1	II	20.0/L	Urine volume for 24 hr.: ——cc.					
0.1	IV	40.0/L	Estrogen assay 24 hr.: ——gamma.					
			(Estrogenic equivalent to estrone)					

Daily examination of smears, for 2 to 4 months from several hundred castrated animals over a period of 2 years, has indicated that the necessary smears for practical use should be 48, 56, 72, 80, and 96 hours after the animal receives its injection. If the animals are injected on Monday A. M. this arrangement calls for smears to be recorded Wednesday A. M. and P. M., Thursday A. M. and P. M., and Friday A. M. If a full estrus reaction is not found during these intervals the result is negative. When estrone is administered the smears taken at the same intervals should also reveal a full estrus reaction or the animal should not be considered controlled. The route of injection must be subcutaneous, both with the standard and the unknown; injections must be single in number and always in the same solvent, olive oil. The use of fewer than 20 animals as stipulated for each test dose in the Report of the Second Conference on the Standardization of Sex Hormones[11] can be satisfactory only if these animals are individually examined and suitably controlled.

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[9] Cohen, S. L., and Marrian, G. F., *Biochem. J.*, 1935, **29**, 1577.

[10] Schmulovitz, M. J., and Wylie, H. B., *Mimeographed Supplement to reference No. 5.*

These studies do not indicate that different groups of mice have a definite constant threshold of reactivity to estrone but rather that by first overstimulation and then persistent adequate stimulation at regular intervals mice can be brought into, and then maintained in, a state of maximal reactivity to a rather constant minimal dose of estrone.

9141 P

Distribution of Phosphorus in the Starch Granule.

Carl L. Alsberg.

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Gelatinized root-starch granules possess a peripheral membrane containing phosphoric-acid ester. This has led some investigators to infer that the natural ungelatinized granule also possesses such a membrane. If this be so, it follows that the P_2O_5 percentage in the small granules of a given sample should be greater than in the large granules, provided the thickness of the hypothetical membrane of the two sorts of granules is about the same.

There are two pertinent observations in the literature. Fernbach[1] found the small-sized granules to contain decidedly more P_2O_5 than the large-sized ones in the same sample of potato starch. He concluded, however, that the granule consists of a nucleus rich in phosphorus upon which are superimposed layers of phosphorus-free material. Kavcic[2] determined the P_2O_5 content of starch from 4 potato varieties with granules of different average diameters. While his figures indicate a tendency for the P_2O_5 percentage to increase as the average granule size decreases, the differences are not as great as they should be considering that the mass of a solid sphere increases as the diameter increases far more than the surface area. The interpretation of such data as these of Kavcic is difficult without knowledge of the frequency distribution of granule sizes in each sample. Another difficulty is the possibility that the P_2O_5 content of starches varies with the variety or the conditions of growth independently of granule size. Mangels[3] has reported such variations for wheat, though he did not correlate them with granule size.

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[11] *Quart. Bull. Health Organization League Nations*, 1935, **4**, 618.

[1] Fernbach, A., *Compt. rend.*, 1904, **138**, 428.

All these difficulties may be avoided by separating each starch sample to be analyzed into fractions of different granule size, and analyzing each fraction separately. The writer had a sample of cassava (tapioca, manioc) starch so separated in the metallurgical laboratory of the university by air flotation in an air classifier. E. Hines analyzed the several fractions by Embden's method for phosphorus. The several fractions had very much the same P_2O_5 content.

It is evident, therefore, that there are starches in which phosphorus content and granule size are not directly correlated. This conclusion is in harmony with the view of Taylor and Schoch[4] that phosphorus is distributed quite irregularly through the granule. Indeed, they believe that the chain molecules of starch "may vary over a wide range with regard to the amount of phosphate held to them, giving rise to a kind of heterogeneity even though all these fractions unlike those from the cereal starches are soluble." Maquenne and Roux[5] also believed that the phosphoric-acid ester of starch (α-amylose or amylopectin) is intimately mixed with the other constituents of the granule.

With the evidence at hand, we are not warranted in inferring that because gelatinized granules possess a peripheral membrane containing phosphoric-acid ester natural ungelatinized granules also possess such a membrane. Indeed, there is no satisfactory evidence that natural granules possess any membrane at all, except remnants of the plastid in which they are formed and these are usually removed in the process of purification. There is, in fact, evidence to the contrary. If α-amylose or amylopectin were localized at the periphery, one would expect those natural granules to have the thickest and strongest membranes that have the greatest phosphoric-acid content. One would expect such granules when boiled to hold together better than sorts with a lesser phosphoric-acid content. As a matter of fact, there is no such relationship between phosphoric-acid content and mechanical resistance to boiling. Potato starch with a fairly high phosphoric-acid content disintegrates rapidly, whereas tapioca starch, also a root starch, but with a much lesser phosphoric-acid content does not. The observations of Lynst-Zwikker,[6] which the writer has been able to confirm, indicate that the membrane of gelatinized granules is an artifact formed during gelatinization by the accumulation of the less soluble materials at the periphery of the granule.

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[2] Kavcic, J., *Kolloidchem. Beihefte,* 1930, **30**, 406.

[3] Mangels, C. E., *Cereal Chem.*, 1934, **9**, 571.

[4] Taylor, T. C., and Schoch, T. J., *J. Am. Chem. Soc.*, 1933, **55**, 4248.

[5] Maquenne, L., and Roux, E., *Comp. rend.*, 1905, **140**, 1303.

9142 P

Differentiation between Vitamin B_4 Deficiency and "Encephalomalacia" in Growing Chicks.

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We[1] described a deficiency disease in growing White Leghorn chicks which was corrected by concentrates of Reader's vitamin B_4. The main symptoms described were lack of growth and incoordination which resulted in extreme difficulty in locomotion. Accompanying the lack of coordination of the muscles and disturbed gait was a tendency of the bird to fall upon its side with the legs in tension and pulled against the abdomen. Examination of the brains of a number of the deficient birds revealed gross lesions in the cerebrum and degeneration which could be observed histologically in both the cerebrum and cerebellum. At that time we stated that the condition encountered was probably the same as that observed in chicks by Pappenheimer and Goettsch[2] and designated by them as an "encephalomalacia." This conclusion was based largely on the histological preparations of the tissues from our animals with the comparison of the histological preparations of the tissues from our animals with the histological results of Pappenheimer and Goettsch.

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[6] Lynst-Zwikker, J. J., *Rec. trav. bot. néerlandaises*, 1921, **18**, 1.

[1] Keenan, J. A., Kline, O. L., Elvehjem, C. A., Hart, E. B., and Halpin, J. G., *J. Biol. Chem.*, 1933, **103**, 671.

[2] Pappenheimer, A. M., and Goettsch, M., *J. Exp. Med.*, 1931, **53**, 11.

During the continuation of our studies on deficiency diseases in chicks we have encountered another type of paralysis, the gross symptoms of which are distinctly different from those observed in vitamin B_4 deficiency. We have observed this condition upon a number of different rations, and have had some success in the development of a ration which will produce the condition in 100% of the birds. Only those rations which allow considerable growth will produce a large incidence of the disease. It is more likely that this condition is the encephalomalacia described by Pappenheimer and Goettsch. Very probably our observations on brain degeneration in what we called vitamin B_4 deficiency were complicated by changes due to encephalomalacia. In vitamin B_4 deficiency growth is checked rather early and the bird succumbs to a vitamin B_4 deficiency before gross symptoms of encephalomalacia appear, although the ration may be low in the encephalomalacia factor. Preliminary histological examination indicates that in severe cases of B_4 deficiency there is no marked degeneration and liquefication of the cerebrum like that which characterizes the encephalomalacia of Pappenheimer and Goettsch. The rations used in producing the B_4 deficiency have been described by O. L. Kline, H. R. Bird, C. A. Elvehjem, and E. B. Hart in the *Journal of Nutrition,* 1936, **11**, 515. The histology of the two distinct deficiency diseases and studies on the properties and the distribution of the two factors will appear in future papers, but in this short note we wish to describe briefly the gross symptoms of the encephalomalacia-like condition and distinguish it from vitamin B_4 deficiency.

The encephalomalacia-like paralysis develops very rapidly. Birds appearing normal in every way may show very severe symptoms in a few hours' time. At first the birds show nervousness and increased activity when disturbed. Soon the animals show a tendency to sit on their hocks and at frequent intervals move backwards pushing with their legs and assuming a penguin-like attitude. If the animal is held and brought near any firm object, it will immediately push against that object with its feet. When completely exhausted the animal will lie on its side still pushing with its feet, but it will never exhibit the tendency to fall to one side or the other like those suffering from vitamin B_4 deficiency.

The accompanying photographs show the distinction in behavior of the birds suffering from the two deficiencies. The encephalo-

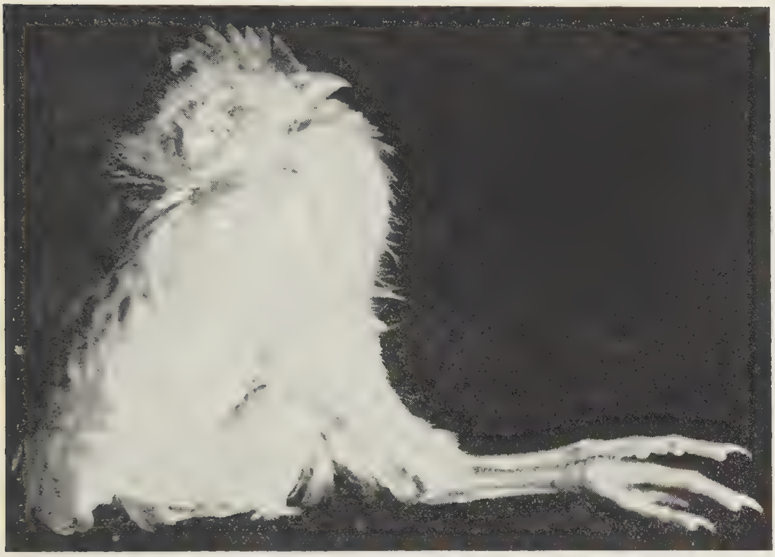

FIG. 1.
A case of "encephalomalacia."

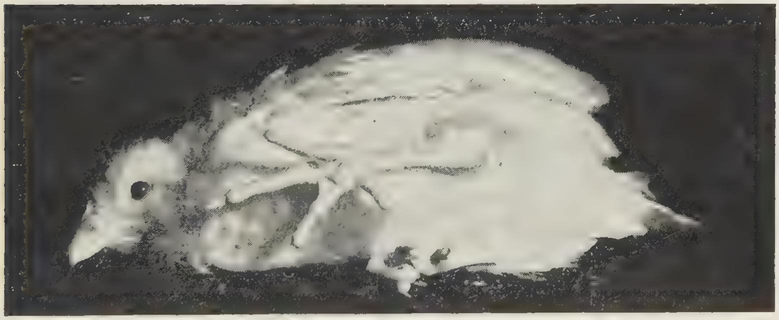

FIG. 2.
A vitamin B_4 deficient chick.

malacia-like condition appears to be very similar to that described by Pappenheimer and Goettsch and also to the condition recently described by Jungherr[3] in birds on certain practical rations.

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[3] Jungherr, E., *Science*, 1936, **84**, 559.

9143 P

Direct Transmission of Human Influenza Virus to Mice.

THOMAS FRANCIS, JR., AND THOMAS P. MAGILL.

From the Laboratories of the International Health Division, Rockefeller Foundation, New York.

Repeated attempts have been made to isolate human influenza virus by the direct inoculation of mice with material obtained from patients acutely ill with the disease. Up to the present, however, the establishment of experimental influenza in mice has been successful only when the virus was first subjected to primary passage in ferrets.

In December, 1936, strains of human influenza virus were isolated from fresh cases of influenza occurring in New York. Following one ferret passage, the strains were transferred to mice and became so rapidly adapted to this species of animal that deaths occurred among the mice of the second passage. The ease with which these results were obtained heightened the possibility of transmitting the virus infection directly from human throat washings to mice without intermediate ferret passage.

With plain meat infusion broth as the medium, nasal and pharyngeal washings were collected from 2 patients acutely ill with influenza. The washings were pooled. One portion was used for ferret inoculation and yielded a strain of virus which was readily transferred from ferret to mice. The remainder was stored in the frozen state at —78°C. for 19 days. It was then removed from storage, allowed to thaw at room temperature and centrifugalized at 2500 revolutions per minute for 30 minutes. The supernatant fluid was then centrifugalized in the air-driven centrifuge[1] at 14,000 r.p.m. for 3 hours. The supernatant fluid was removed, the sediment was resuspended in the supernatant to 1/6 the original volume and anesthetized mice were then given 0.05-0.08 cc. of the material intranasally.

Serial passages were made in mice at 4 to 5-day intervals. A heavy suspension (50 to 70%) of the finely ground lung tissue from inoculated mice was given intranasally to normal mice. During the first 3 passages no significant pulmonary lesions were observed. It was shown, however, that virus was present in the lungs of the third passage mice, since a ferret inoculated intranasally with a suspension of these lungs developed the characteristic signs of experimental influenza and subsequently developed neutralizing antibodies against human influenza virus. In the 4th passage, the lungs of all the mice presented small areas of involvement. Thereafter, a rapid increase in the virulence of the infectious agent occurred and fatal infection ensued among mice of the 6th passage. For the 7th passage a bacterium-free Berkefeld V filtrate was used. Mice of the 8th passage began to die on the 4th day after their inoculation with a 10% suspension of the involved lung tissue. Neutralization tests, using suspensions of the lungs of mice of the 7th serial passage and known immune serum, clearly identified the infectious agent as human influenza virus. The results are summarized in Table I.

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[1] Bauer, J. H., and Pickels, E. G., *J. Bact.*, 1936, **31**, 53.

TABLE I.

Direct transfer of human influenza virus from throat washings of influenza patients to mice.

Passage No.	Date of autopsy	Lung lesions	Remarks
1	1/ 3/37	0, +, 0, 0, 0	
2	1/ 7/37	0, 0, 0, 0, 0, 0	
3	1/11/37	0, 0, 0, 0, 0, 0	Ferret inoculation positive
4	1/16/37	+++, +, +, +, +, +	
5	1/20/37	+++, ++, ++, ++, ++, ++	
6	1/23/37	++++,* ++++, ++++, ++, ++++, +++	
7 (Filtrate)	1/28/37	+++, +++, +++, +++, ++, ++	Neutralization test done
8	2/ 1/37	++++,* ++++,* ++++,* ++, ++	

Patients: BL 3 and 4. 12/10/36. Original mouse inoculation 12/29/36.
0 to ++++ indicate progressive degrees of lung involvement.
* indicates death of mouse.

It has been possible, therefore, to establish infection with human influenza virus in mice by the direct intranasal inoculation of these animals with throat washings obtained from patients acutely ill with influenza. It is apparent, however, that the procedure as described still lacks certain advantages which accrue to the use of the ferret in which the prompt febrile reaction quickly indicates the presence of active virus. In the case of mice, a comparatively long period exists during which the observer has little or no objective evidence of the presence or absence of virus. Under other conditions, or with other strains, this long period may be shortened or even eliminated.

9144 P

Direct Isolation of Human Influenza Virus in Tissue Culture Medium and on Egg Membrane.

Thomas Francis, Jr., and Thomas P. Magill.

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Throat washings in Tyrode's solution were obtained on the second day of illness from a patient acutely ill with influenza. The material was centrifugalized at 2500 revolutions per minute for 30 minutes. The supernatant fluid was then filtered through a graded collodion membrane of 500 mμ average pore size.[1] Flasks containing 4.5 cc. of chick embryo Tyrode medium[2] were inoculated with 2.5 cc. amounts of the filtrate (shown by ferret inoculation to contain active virus). Transfers of 0.5 cc. amounts of the culture material to 4.5 cc. of fresh medium were made at 48-hour intervals. Mice were inoculated intranasally with culture fluid of the 5th transfer. Mouse passages were made at 4-day intervals. No significant lesions were seen in the lungs of mice of the first 3 passage groups. In mice of the 4th passage, however, sugestive lesions were seen; these were more definite in the 5th passage, and in the 6th passage death of the mice with extensive pulmonary involvement occurred. The virus was identified as human influenza virus by means of neutralization tests with known immune serum.

These results indicate that virus was recovered directly from the throat of a human influenza patient by the introduction of the filtered throat washings of the patient into tissue culture medium. The virus not only survived but probably multiplied, since the final dilution of the original material was approximately 1:300,000 at the time the culture material was first given to mice. The behavior of the virus after its inoculation into mice was very similar to that of virus established directly in mice from human throat washings.[3]

Burnet[4] has reported in detail the behavior of human influenza virus introduced after passage through ferrets, onto the chorio-allantoic membrane of the developing chick. The direct cultivation of the virus of common cold on the chorio-allantoic membrane has also been reported.[5] Attempts were therefore made to utilize this procedure in the isolation of virus directly from the human patient. Throat washings in broth were obtained from two patients acutely ill with influenza. By means of ferret inoculation and subsequent adaptation to mice, virus was shown to be present in the material. Portions of the throat washings of these patients were pooled, centrifugalized at 2500 r.p.m. for 30 minutes and filtered through a graded collodion membrane of 500 mμ average pore size. The chorio-allantoic membrane of a 12-day chick embryo was inoculated with 0.05 cc. of the filtrate. Passages were made at 4-day intervals by inoculating additional embryonic membranes with finely ground emulsions of the previous passage membranes. Thickening of the membranes was observed and white plaque formations were seen. A suspension of the 3rd passage membrane was inoculated into a ferret and into mice. The ferret developed typical experimental influenza and its convalescent serum neutralized human influenza virus. No lesions were seen in the lungs of mice of the first 3 passages. In those of the 4th, 5th, and 6th passages small pulmonary lesions were seen, but they have not been sufficiently extensive to permit of conclusive neutralization tests.

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[1] Bauer, J. H., and Hughes, T. P., *J. Gen. Physiol.*, 1934, **18**, 143.

[2] Li, C. P., and Rivers, T. M., *J. Exp. Med.*, 1930, **52**, 465.

[3] Francis, T., Jr., and Magill, T. P., Proc. Soc. Exp. Biol. and Med., 1937, **36**, 132.

[4] Burnet, F. M., *Brit. J. Exp. Path.*, 1936, **17**, 282.

After the 3rd transfer on egg membranes the virus was placed in storage for several days. Since that time the changes in the passage membrane have been less pronounced. These brief observations indicate, however, that human influenza can be isolated directly and maintained for a time, at least, upon the embryonic membranes of developing chick.

The practicability of both the procedures described above is being studied on a larger scale. Certain disadvantages obtain. These consist in the inability to demonstrate the presence of influenza virus in tissue culture medium except by establishing the virus in animals and in the fact that the lesions produced by the virus in the early passages on embryonic membranes are too irregular to enable one to identify the virus. It seems possible that future studies will furnish means of eliminating such difficulties.

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[5] Kneeland, Y., Jr., Mills, K. C., and Dochez, A. R., PROC. SOC. EXP. BIOL. AND MED., 1936, **35**, 213.

9145 P

The Adrenals and Pituitary in Initiation of Lactation.

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The authors[1] have shown, and Gomez and Turner[2] confirmed, that a purified pituitary lactogenic extract, unlike crude pituitary extract, will not produce lactation in hypophysectomized guinea pigs, but will do so if adrenal cortical hormone is simultaneously administered. A further analysis of factors permitting lactation in the guinea pig is reported here.

Mammary glands of guinea pigs, male or female, were developed by oestrin* injections of 30 or more R.U. per day for not less than one month. Purified lactogenic hormone* was used in doses of 10 mg. or more of powder daily. Glucose solutions were injected after hypophysectomy.

I. It was again found that lactogenic hormone would not, after 8 days' treatment, start lactation in hypophysectomized pigs (3 cases). It would, however, cause lactation if cortical hormone* were given simultaneously (3 cases).

II. That either crude pituitary extract alone or purified lactogenic hormone plus cortical hormone will induce lactation, suggested that the adrenotropic fraction is the missing factor in purified extracts. This assumption was verified by experiments in which 4 hypophysectomized pigs were treated with lactogenic hormone plus 2 cc. daily of adrenotropic hormone.* All animals lactated within 1-3 days. One of these pigs was treated for a week with lactogenic hormone, without lactating, before treatment with adrenotropic hormone was begun. Another was treated 6 days with adrenotropic hormone, without lactating, before treatment with lactogenic hormone was begun. A fifth animal, treated for a week with adrenotropic hormone only, failed to lactate. These facts suggest that adrenotropic and lactogenic fractions are not identical.

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[1] Nelson, W. O., and Gaunt, Robert, Proc. Soc. Exp. Biol. and Med., 1936, **34**, 671.

[2] Gomez, E. T., and Turner, C. W., Proc. Soc. Exp. Biol. and Med., 1936, **35**, 365.

* This work was made possible by gifts of the following materials, for which the authors are indebted: Adrenal cortical extract—Dr. W. W. Swingle, Princeton University; crude pituitary extract (sheep), lactogenic hormone and theelin—Dr. Oliver Kamm, Parke-Davis and Co.; amniotin—Dr. J. A. Morrell, E. R. Squibb and Sons; adrenotropic hormone—Dr. J. B. Collip, McGill University.

III. Since salt therapy is known to alleviate symptoms of adrenal insufficiency, attempts were made to induce lactation in hypophysectomized pigs treated with lactogenic hormone and high doses of NaCl. Salt was given by subcutaneous or intraperitoneal injection, in drinking solution, or by stomach tube, in amounts regulated to prevent diarrhea. Any type of salt therapy tried plus purified lactogenic hormone induced lactation within 4-5 days (7 cases).

IV. Attempts to induce lactation in adrenalectomized pigs were difficult because the guinea pig is quickly susceptible to adrenal insufficiency, and crude pituitary extracts hasten death. Pigs were treated for 36 hours after adrenalectomy with cortical extract and salt to assist in recovery from the severe operation. Twenty-four hours after the last cortical hormone injections, lactogenic extracts were begun, and salt therapy continued. Eleven cases of complete adrenalectomy were thus obtained which lived from 3 to 12 days after the beginning of lactogenic injections. None lactated. Five of these had received purified lactogenic hormone, living an average of 8 days after lactogenic hormone injections began. Six received crude pituitary extract, living an average of 4.6 days. With longer survival it is possible, but improbable, that salt-treated, adrenalectomized pigs on crude extract might eventually have lactated.

In completely adrenalectomized pigs, if cortical extract and pituitary extract were given throughout the period after adrenalectomy (one case) or after pituitary extracts alone had failed to induce lactation (3 cases) milk secretion started.

Incompletely adrenalectomized pigs lactated within 60 hours after either crude or purified lactogenic extracts were given (6 cases).

An amount of cortical hormone, or tissue, insufficient to prevent adrenal insufficiency (asthenia, weight loss, anorexia) or even to maintain life, will generally permit lactation. This was unexpected because the hypophysectomized pig, without any marked symptoms of adrenal insufficiency, must nevertheless have more cortical hormone than its own adrenals will release in order to lactate. The theory that lactation failure in adrenalectomized animals is due primarily to disturbances in fluid and electrolyte balance, which explains satisfactorily the phenomenon observed in adrenalectomized lactating rats,[3, 4] and in salt-treated hypophysectomized guinea pigs is

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[3] Gaunt, Robert, and Tobin, C. E., *Am. J. Physiol.*, 1936, **115**, 588.

[4] Levenstein, Irving, *Anat. Rec.*, 1937, (in press).

less well illustrated, but not precluded, in adrenalectomized pigs. In the latter animals clear serum was nearly always expressible from the nipples during the observation period. If cortical hormone or tissue was present this fluid quickly became milk. Sufficient fluid for milk flow was probably present.

In guinea pigs lactating normally after parturition, production of "artificial adrenal insufficiency"[5] by intraperitoneal injection and subsequent withdrawal of isotonic glucose solution for 2 successive days, either entirely or almost, stopped lactation (4 cases). Lactation was quickly reinstated by salt solutions given orally.

Conclusion. Experiments of several types indicate that adrenal cortical hormone is necessary for milk production, and that the disturbance of cortical function produced by hypophysectomy, or adrenalectomy prevents the initiation of lactation in the guinea pig.

9146

Horizontal Respiratory Graphs by Automatic Feed of Oxygen into a Basal Metabolism Apparatus.

MORRIS ECKMAN AND ALVAN L. BARACH.

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When respiratory tracings are taken of a subject breathing air, oxygen must be added to maintain the oxygen concentration at the desired level. A previous method employed a calibrated air pump by which the experimenter forced into the gas circuit sufficient oxygen to maintain the graph approximately horizontal. The oxygen concentration in the circuit was subject to considerable variation depending on the frequency with which the oxygen consumed by the patient was replenished. Furthermore, the graphs were difficult to calculate accurately, since the oxygen admitted was reflected intermittently on the tidal air curve. The "concertina apparatus" was used by Haldane[1] for gases of fixed concentrations but the expiratory phase on the graph was traced as a result of the refilling of the concertina from a gas source other than the lungs. Poulton[2] employed an apparatus in which the volume of air in the circuit remained constant, oxygen replacing the CO_2 absorbed by the soda lime.

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[5] Gilman, Alfred, *Am. J. Physiol.*, 1934, **108**, 662.

[1] Haldane, J. S., "Respiration," new edition, University Press, London, 1935, p. 208.

[2] Poulton, E. P., *Guys Hospital Gazette*, 1935, **49**, 346.

By means of the levelling device described below, a horizontal graph of the tidal air is obtained with minimal fluctuations of the oxygen content, ±0.5%, and without artificial disturbance of the patient's pulmonary ventilation.

The levelling bulb (1), half filled with water, is fastened to the spirometer bell with the base of the bulb resting on top of the bell. The levelling device is adjusted to a height that permits slight immer-

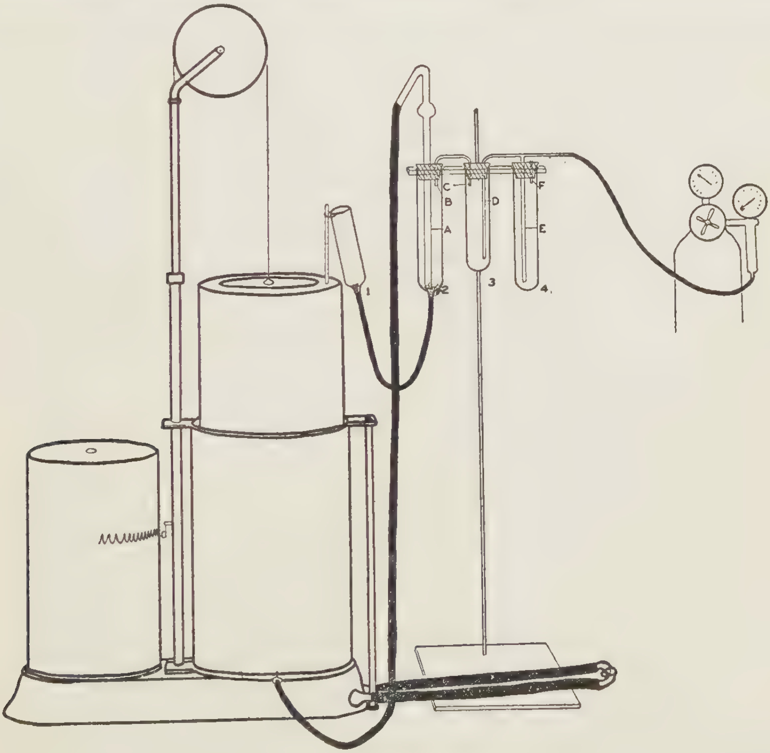


FIG 1

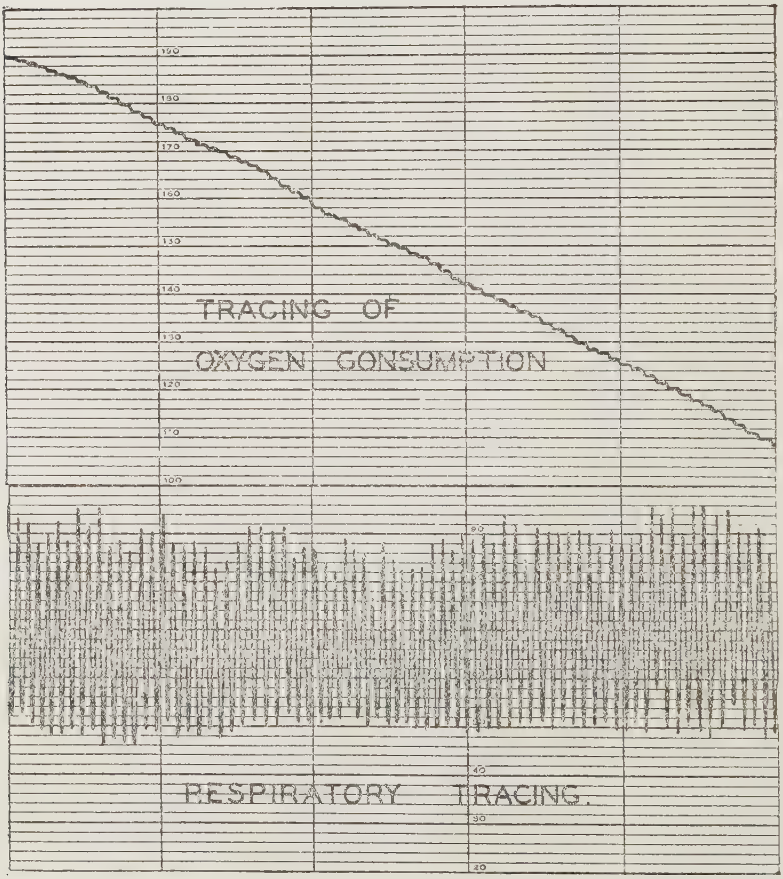
Description of apparatus for text figure 1.

(1) Levelling bulb joined by thin-walled rubber tube to (2) water valve which contains tube A. This tube, which is inserted to within 2 mm. of the bottom of the water valve, has a bulb (2.5 cm. I.D.) blown in its length 25 cm. from the bottom to prevent water from being carried into the spirometer circuit. The water valve is connected by delivery tube B-C (5 mm. I.D.) to the (3) water trap which contains tube D (5 mm. I.D. x 14 cm. to bend.) which is inserted to within 2.5 cm. of the bottom of the trap and connected to one horizontal arm of the T tube E. The vertical arm of tube E is inserted to within 2.5 cm. of the bottom of the (4) constant pressure trap. An oxygen escape tube F is run through the stopper of the pressure trap to permit escape of excess oxygen. The oxygen supply at 3 liters per minute enters the remaining arm of the T tube E.

sion of tube A when the spirometer bell is half filled. The subject's lungs and the apparatus are first washed out with the gas mixture to be breathed; the bell is then filled half way, the recording kymograph drum set off and an oxygen flow of 3 to 4 liters per minute started through the levelling device.

As oxygen in the basal metabolism apparatus is consumed, the spirometer bell falls until the water level in the water valve (2) sinks beneath the lower end of tube A. Oxygen will immediately enter the gas circuit through tube A and raise the bell until the bottom of the tube is again immersed in water. Thus a sufficient flow of oxygen will be admitted to balance the oxygen consumption and keep both the bell level and the oxygen concentration constant.

The water trap (3) should contain enough water to immerse



GRAPH 1

tube D to a depth of 1 cm. Its function is to prevent any gas from the basal metabolism apparatus from escaping through the levelling device.

The constant pressure trap (4) should contain enough water to immerse tube E to a depth of 5 cm. This trap insures a constant supply of oxygen at 5 cm. pressure to the water valve (2). Excess supply of oxygen to tube E leaves the bottom of the tube.

If a record of oxygen consumption is required, as in determining oxygen deficits, a spirometer filled with oxygen, weighted to deliver gas at 5 cm. pressure and containing a volume recording device may be connected to the levelling device in place of the constant pressure trap (4). A simultaneous graphic record of respiration and oxygen consumption (Graph I) will thus be obtained.

Summary. The levelling device described makes it possible to record horizontal respiratory graphs by means of a continuous inflow of oxygen, automatically regulated to correspond to the patient's oxygen consumption. Any gas mixture desired may be kept constant during the test period since the oxygen concentration in the basal metabolism circuit remains within ±0.5% of the original concentration. With the use of an auxiliary recording spirometer, it is possible to record oxygen consumption and respiratory tracings simultaneously. Oxygen deficit may be accurately recorded.

9147

The Blood Picture in the Alarm Reaction.

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It has recently been shown that if the general condition of an animal is seriously injured by acute, non-specific damaging influences, such as exposure to cold, surgical injuries or intoxication with sublethal doses of various drugs, a characteristic symptom complex appears which has been called the "alarm reaction". It is probably the expression of the defence of the organism against damage as such. The anatomical changes seen in this syndrome have been described in previous reports.[1, 2] The principal ones are thymus, spleen and lymph gland atrophy; marked tendency towards

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[1] Selye, H., *Nature*, 1936, **138**, 32.

[2] Selye, H., *Br. J. Exp. Path.*, 1936, **17**, 234.

edema formation; adrenal enlargement; and gastric and intestinal ulcers.

The severe changes in the lymphatic system made it likely that the alarm reaction would also influence the white cell count of the blood. The demonstration of constant changes in the blood picture seems desirable, because they would also be of diagnostic significance for the recognition of the alarm reaction during life.

Numerous workers have studied the effect of exercise, surgical shock, cold and drugs on the leucocyte count of the blood. Leake[3] found that subcutaneous injections of morphine caused a polymorphonuclear neutrophilic leucocytosis. Congo[4] also showed an increase in the neutrophils after treatment with pilocarpine, eserine and adrenalin. Josey and Lawrence[5] observed a moderate neutrophilic leucocytosis in the guinea pig after prolonged exposure to cold. Schultz[6] noted that after operative procedures there was an increase in the neutrophilic leucocyte count, and Weindrach[7] found a similar increase in the human after severe exercise. Borchardt[8] observed that the leucocytosis usually produced by drugs (pilocarpine, choline) in the cat is not seen after adrenalectomy, but since the adrenalin leucocytosis is also prevented by suprarenalectomy he does not agree with previous investigators who considered nonspecific leucocytosis to be the result of increased adrenalin secretion.

In order to determine whether the above mentioned changes are specific or simply the result of slight alarm reactions, we studied the blood picture changes following exposure to various damaging agents coincident with the symptoms of the alarm reaction caused by such stimuli. These experiments, which we should like to report here, allowed us to determine the repercussion of the alarm reaction on the blood count.

Both rats and mice were used for our experiments; the former were 2½ months old, the latter 2 months old. The blood of each experimental animal was examined both before and after the experiment, in order to avoid errors due to individual variations in the white cell count. Eight animals were used in each series.

In the first series, an alarm reaction was elicited by means of drugs such as adrenalin and formaldehyde. Adrenalin was given subcutaneously twice daily, the rats receiving 0.2 mg., the mice 0.15 mg.

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[3] Leake, C. D., *J. Am. Med. Assn.*, 1922, **78**, 1687.

[4] Congo, W. J. R., *J. Lab. and Clin. Med.*, 1927, **13**, 206.

[5] Josey, A. I., and Lawrence, J. S., *Fol. Haematol.*, 1932, **48**, 303.

[6] Schultz, G., *Deutsch. Arch. f. klin. Med.*, 1893, **51**, 234.

[7] Weindrach, G., *Fol. Haematol.*, 1929, **38**, 276.

[8] Borchardt, W., *Naunyn-Schmiedebergs Arch.*, 1929, **139**, 47.

per dose. Formaldehyde was given subcutaneously twice daily in doses of 0.5 cc. of a 4% solution to rats, and 0.2 cc. of the same solution to mice. Forty-eight hours after the initial injection, there was lymphopenia with marked leucocytosis due to an increase in the number of polymorphonuclear-neutrophiles. Lethal doses of formaldehyde, on the other hand, caused leucopenia which became so marked that in the terminal stages white cells had almost completely disappeared from the blood.

In the second series, the alarm reaction was produced in rats and mice by exposure to cold. The rats were placed into an icebox at a temperature of 0°C. for 5 hours a day on 2 consecutive days. Since their fur protects them very well from cold, we found it advisable to wet their skin before the exposure. In the case of the mice this was not necessary, and exposure to 0°C. twice daily for 3.5 hours on 2 consecutive days was found sufficient to elicit an alarm reaction. Forty-eight hours after initiation of treatment, they showed leucocytosis and lymphopenia. Counts made immediately after the last exposure to cold, at a time when the animals were obviously seriously damaged, showed an inverse response—that is, leucopenia.

In the third series, an alarm reaction was elicited by means of surgical shock. This was produced in the rats by gentle handling of the intestines exposed aseptically through a small abdominal incision for 4 minutes, and in the mice for 1.5 minutes. Forty-eight hours after the operation, leucocytosis again due to increase in neutrophiles was observed.

In the fourth series, the alarm reaction was elicited by means of excessive muscular exercise in a revolving cage having a diameter of 12 inches and running at 18 revolutions per minute. The rats

TABLE I.
Average white cell count in the alarm reaction.

	Normals	Formaldehyde	Adrenalin	Cold	Surgical Shock	Exercise
			Rats			
Total white cell count	13,034	25,264	22,700	22,800	25,800	24,600
Polymorphs	35	76	71	78	84	64
Small Lympho.	58	17	24	14	11	31
Large Lympho.	7	7	5	8	5	5
			Mice			
Total white cell count	8,832	18,540	20,010	13,300	17,540	16,800
Polymorphs	38	69	68	68	63	58
Small Lympho.	56	25	26	26	31	33
Large Lympho.	6	6	6	6	6	9

were exercised for 0.5 hr. twice daily the first day, and 0.75 hr. twice daily the second day; the mice 0.5 hr. twice daily on two consecutive days. Forty-eight hours after the initiation of the experiment, marked neutrophilic leucocytosis was observed. Our findings are summarized in Table I.

Summary. We conclude that the total white cell count increases mainly as a result of neutrophilic leucocytosis during the alarm reaction. At the same time, there is relative lymphopenia. Following exposure to very severe alarming stimuli, a period of leucopenia precedes this characteristic reaction. Leucopenia is observed throughout the whole experiment in those cases in which the animal was unable to resist the alarming stimulus so that death ensued.

9148

Effects of Ovariectomy on Body Growth and Organ Weights of the Young Albino Rat.

CLAY B. FREUDENBERGER AND PHILIP M. HOWARD.
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Freudenberger and Billeter[1] studied the effect of spaying on body growth and the organ weights of the albino rat. In their series the ovaries were removed from the experimental animals at an average age of 26 days. All animals were completely autopsied at 6 months of age. Differences in body growth between experimental and control rats as well as many differences in organ weights were noted. Lawless[2] has recently called attention to the fact that observations published regarding the effects of castration usually concern only that characteristic which it is sought to influence, so that the published reports do not furnish a complete picture of all the changes in a castrated animal. He stated that a study of all the observable changes resulting from castration in one species would therefore seem desirable. These two statements apply equally well to the studies concerning the effects of bilateral ovariectomy. We are, therefore, carrying our studies further on this problem. We wish to present here the results of bilateral ovariectomy on young albino rats as noted when the animals were about 3 months of age. Only brief mention will be made of the literature, since the more important articles have been referred to by recent writers.[1, 3]

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[1] Freudenberger, C. B., and Billeter, O. A., *Endocrinology*, 1935, **19**, 347.

[2] Lawless, J. J., *Anat. Rec.*, 1936, **66**, 455.

[3] Holt, H., Keeton, R. W., and Vennesland, B., *Am. J. Physiol.*, 1936, **114**, 515.

The rats used in our work were of the Wistar strain. At the time of operation the average age of both test and control animals was 26 days with a range of from 23 to 29 days. Twenty-four test animals and 24 controls were used. In most cases the animals used for tests and controls were litter mates. The average weight at the time of operation of both the test and control animals was 47 gm. Excellent care was given all animals, the diet being the same as that used by Freudenberger and Billeter.[1] The ovaries were removed through a midline abdominal incision. In the animals used as controls the incision was made but the ovaries were only slightly disturbed, thus assuring approximately the same operative conditions. No infections occurred in any of the animals. All animals were weighed at weekly intervals. At an average age of 93.3 days (ranging from 91 to 96 days), both the ovariectomized and control animals were killed in a gas chamber. Each animal was autopsied immediately after it was killed, and the organs were then weighed. The method of autopsy was essentially the same as that used by Jackson.[4] All conditions were very similar to those that prevailed at the time the previous experiment concerning spaying was done in this laboratory, so we feel that the results should be comparable. Modern biometrical methods were again used in interpreting the results.

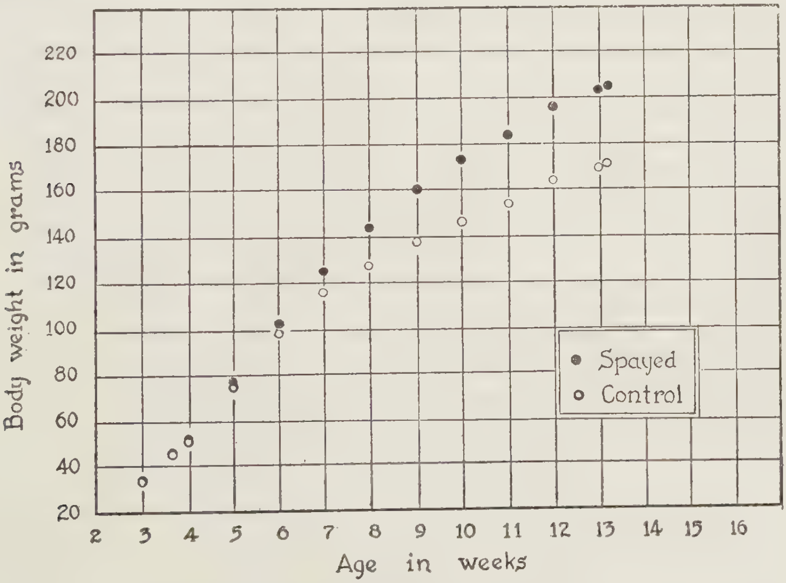

FIG. 1.
Average body weight in grams according to age in weeks.

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[4] Jackson, C. M., *J. Nutrition*, 1930, **3**, 61.

As stated recently by Holt, Keeton, and Vennesland,[8] most workers have reported that ovariectomized animals gain more weight than controls. This was found to be true in our series (Fig. 1). At 5 weeks of age the test animals had an average weight which was 3% greater than that of the controls. At 6 weeks of age this difference in average weights amounted to 4%. The difference at 7 weeks was 8.8%, and this difference was found to be significant. The percentage difference in body weight between the tests and controls constantly increased until 13 weeks of age when the difference was 20.5%. At the time of autopsy the ovariectomized animals had an average weight of 205.4 gm. (range: 171-242 gm.) and the controls had an average weight of 170.9 gm. (range: 155-195 gm.). Thus, the average weight of the tests was 20.2% greater than that of the controls.

It was noted that the test animals became significantly heavier than the controls shortly after the latter became sexually mature. The vagina opened in the control rats at an average age of 42 days, and the difference in body weight between the 2 groups was found to have become significant at an average age of 49 days. However, as stated above, the test rats weighed more than the controls at 35 days of age.

TABLE I.
Organ Weights, Differences and Significance Ratios
(Measurements in grams and centimeters).

	Mean Tests	Mean Controls	Difference	% Difference	Significance Ratio
Body weight	250.4	170.9	+34.5	20.2	11.5
Body length	19.40	18.83	+ 0.57	3.1	7.0
Tail length	18.77	17.91	+ 0.86	4.8	6.5
Head	17.78	15.79	+ 1.99	12.6	10.3
Hypophysis	0.0104	0.0088	+ 0.0016	18.2	6.4
Thyroid	0.0200	0.0181	+ 0.0019	10.5	3.3
Suprarenal glands	0.0376	0.0416	— 0.0040	10.6	3.6
Thymus	0.794	0.450	+ 0.344	76.5	10.1
Heart	0.809	0.714	+ 0.095	13.3	6.9
Lungs	1.505	1.283	+ 0.222	17.3	4.5
Alimentary group	17.0	14.5	+ 2.5	17.3	6.3
Stomach	0.897	0.758	+ 0.139	18.4	10.2
Liver	7.607	6.763	+ 0.845	12.5	4.9
Spleen	0.516	0.418	+ 0.095	22.6	18.6
Kidneys	1.561	1.442	+ 0.119	8.2	3.8

Our findings are shown in Table I. The ovariectomized rats had a greater body length, tail length and head weight than the controls. The average weights of all the organs studied were also greater in the tests than in the controls with the one exception of the suprarenal glands. All of these differences were significant as indicated by the significance ratios which varied from 3.3 for the thyroid to 18.6 for the spleen. The average weight of the suprarenal glands of the test animals was less than that of the controls. However, the difference in the weights of the suprarenal glands was not nearly so great as that found at 6 months of age. Although the percentage differences between the body weights of the test and control animals were only slightly greater at 3 months than at 6, the differences in organ weights were much greater. This would indicate that the body length and the organs of the ovariectomized rats underwent their growth changes more rapidly than those of the controls even though their final values were in many cases no greater than in normal rats. Holt, Keeton, and Vennesland[3] found that the percentage of dry matter, nitrogen, ash, and fat in gonadectomized, eviscerated female carcasses, to which the visceral fat had been added, fell within the normal ranges. They, therefore, concluded that the increase in body weight in spayed animals was due to a general increase in size rather than to any increase in the deposition of fat. They stated that the conclusion seemed warranted that ovariectomy influenced also the growth-promoting properties of the pituitary. Our results concerning body lengths and organ weights of ovariectomized rats also tended to confirm this theory. However, we had no proof that this was the true explanation of the results. It was interesting to find that R. G. Hoskins[5] in 1911 in commenting on the hypertrophy of the hypophysis occurring in both sexes of the rat following gonadectomy made the following statement: "These observations suggest that the pituitary may normally be held in check by secretions of the gonads, and that when this inhibition is removed, the pituitary manifests increased activity, leading to altered metabolisms and thus to overgrowth of different parts of the body, such as occurs both in acromegaly, and after castration."

Although we did not attempt to measure the amount of fat in our rats we felt that part of the difference in weight between spayed and normal rats was due to a greater deposition of fat in the spayed animals. While this was our impression in rats at both 3 and 6 months of age, it was more marked in the 6 months rats in which the organ weight differences were not so great but the difference in body weight was still present.

Summary. The ovaries were removed from 24 Wistar albino rats which averaged 26 days of age. An equal number of litter mate controls were used. All rats were kept under similar conditions and were killed and autopsied at 3 months of age. The endocrine glands were studied as well as certain other organs which showed some tendency to be changed in a 6-month group of spayed rats which were studied earlier in this laboratory. The vagina opened in the control rats at an average age of 42 days. The body weight of the spayed rats became significantly greater than that of the controls when they were between 6 and 7 weeks of age. In this group of rats the percentage difference in body weight between the spayed and controls constantly increased until 13 weeks of age when the difference was 20.5%. The spayed rats at 3 months of age had a significantly greater body length and tail length than the controls. The following parts were significantly heavier in the spayed rats: head, hypophysis, thyroid, thymus, lungs, heart, alimentary group, stomach, liver, spleen, and kidneys. These differences were all greater than those found at 6 months, which seemed to indicate that ovariectomized rats go through their growth changes more rapidly than controls. The suprarenals were significantly smaller in the spayed rats, but the difference was less than at 6 months.

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[5] Hoskins, R. G., *Am. J. Med. Sci.*, 1911, **141**, 535.

9149 P

Action of P-Aminobenzenesulfonamide on Type III Pneumococcus Infections in Mice.

Frank B. Cooper, Paul Gross and R. R. Mellon.

From the Institute of Pathology, The Western Pennsylvania Hospital, Pittsburgh.

The discovery of prontosil by Domagk[1] has resulted in a new and very effective means of treating certain infections caused by hemolytic streptococci. Hörlein,[2] in reviewing Domagk's work, claimed that this compound was also effective against Type III pneumococcal infections, but failed to publish in detail the experimental results which led either to this conclusion, or to his grouping the Type III pneumococci with the streptococci on chemotherapeutic grounds.

The work of Tréfouël and his associates,[3] confirmed by Buttle, Gray, and Stephenson,[4] indicated that *p*-aminobenzenesulfonamide, a part of the prontosil molecule, was as effective against hemolytic streptococcal infections as the more complicated compounds. Colebrook and Kenny[5] and the present authors[6] have found *p*-aminobenzenesulfonamide even more efficacious than prontosil.

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[1] Domagk, G., *Deutsche med. Wchnschr.*, 1935, **61**, 829.

[2] Hörlein, H., *Proc. Royal Soc. Med.*, 1936, **29**, 321.

[3] Tréfouël, J., and Tréfouël, J. Mme., Nitti, F., Bovet, D., *Compt. Rend. d. Soc. de Biol.*, 1935, **120**, 756.

[4] Buttle, G. A. H., Gray, W. H., and Stephenson, D., *Lancet*, 1936, **1**, 1206.

In view of these results, the therapeutic effectiveness of the *p*-aminobenzenesulfonamide* was determined against Type III pneumococcal infections in mice.

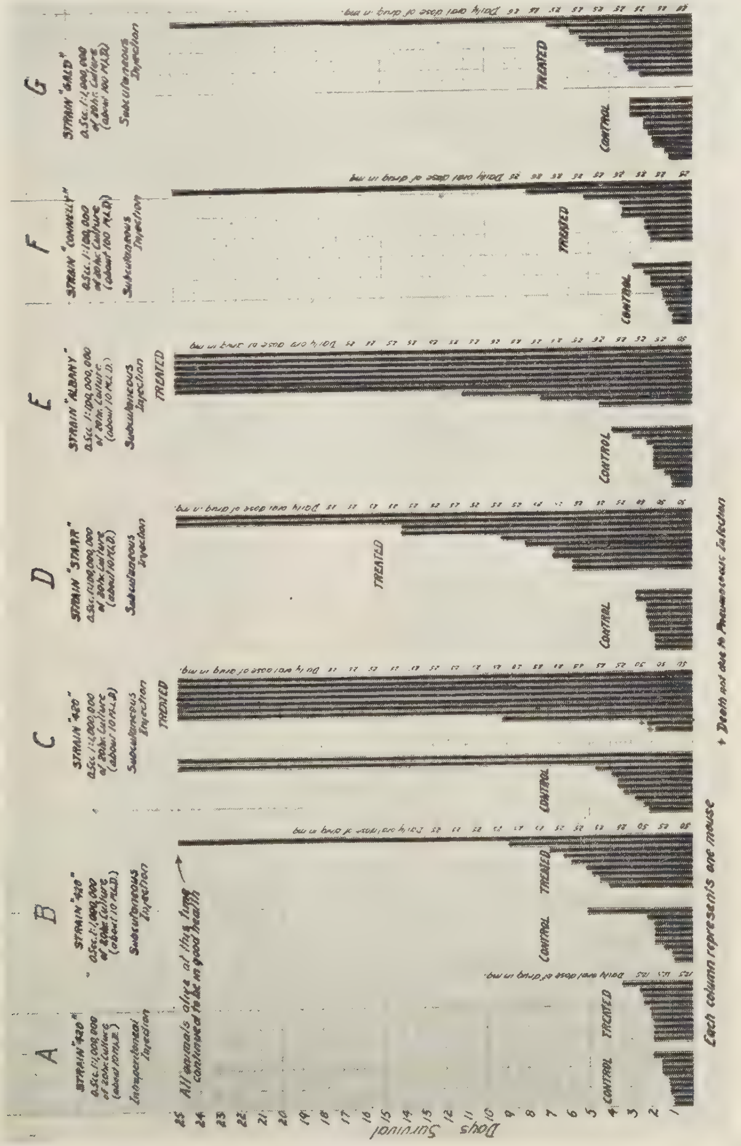

FIG. 1.

Graphic representation of results obtained by the oral administration of *p*-aminobenzenesulfonamide in mice infected with various strains of Type III pneumococcus.

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[5] Colebrook, L., and Kenny, M., *Lancet*, 1936, **2**, 1319.

[6] Mellon, R. R., Gross, P., and Cooper, F. B., *J. Am. Med. Assn.*, in press.

* Kindly supplied by Winthrop Chemical Company, Inc., New York City.

In a preliminary experiment, 19 mice were inoculated intraäbdominally with approximately 10 minimal lethal doses of Strain 420 of Type III pneumococcus. Ten of the mice were given 12.5 mg., of the drug by mouth immediately after infection and every 24 hours thereafter; while the remaining 9 mice received no treatment and served as controls. A negligible prolongation of the survival time of the treated group was obtained. The data are shown graphically in *A*, Fig. 1.

A second series of 18 mice were given the same infecting dose by the subcutaneous route, and half of them treated as indicated in *B*, Fig. 1. Reference to this chart shows a marked increase in the survival-time of the treated group.

A third series of 20 mice (*C*, Fig. 1) were infected by the subcutaneous route with the same dilution of the same 420 culture which had been allowed to deteriorate in virulence. The untreated animals, with the exception of one survivor, lived approximately twice as long as the controls of the previous experiment *(B)*; whereas 7 of the treated animals were alive after 25 days. Two of the treated animals died from causes other than the pneumococcal infection, while only one died of pneumococcal septicemia.

Additional experiments with 4 other Type III pneumococcal strains were conducted. Three of these (*D*, *F*, and *G*, Fig. 1), had recently been isolated from the sputum of fatal pneumonia cases. All experiments showed a definite increase in the survival-time of the treated mice. The "Albany" strain (*E*, Fig. 1), which was a stock strain, showed a 70% survival of the treated mice after 25 days.

Buttle, Gray, and Stephenson[4] have shown that *p*-aminobenzenesulfonamide was effective in bringing about recovery in mice previously infected with 10,000 lethal doses of a highly virulent hemolytic streptococcus. Our own work[6] has shown a similar high protective and therapeutic effect against experimental hemolytic streptococcal infections. Proom[7] was able to protect mice under optimal conditions against one million fatal doses of meningococci by means of this drug.

The action of *p*-aminobenzenesulfonamide in combatting Type III pneumococcal infections in mice does not appear at first to be as effective as its action on either streptococcal or meningococcal infections. The results obtained against subcutaneous Type III pneumococcal infections of less than 10 minimal lethal doses are sufficiently striking, whereas little protection is shown against heavier infecting doses. However, when the extreme susceptibility of mice to pneumococci is considered, the degree of protection demonstrated possibly assumes a greater magnitude, even though the number of fatal doses against which protection was obtained is not so high numerically as in streptococcal or meningococcal infections. The results of these experiments with mice justify a therapeutic trial of *p*-aminobenezenesulfonamide in human Type III pneumococcal infections.

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[7] Proom, H., *Lancet*, 1937, **1**, 16.

Conclusions. The oral administration of *p*-aminobenzenesulfonamide is capable of prolonging, and in some cases of saving, the lives of mice infected subcutaneously with approximately 10 minimal lethal doses of highly virulent Type III pneumococci.† ‡

9150

Influence of Deuterium Oxide on Growth and Morphology of Lactobacilli.

HARRY H. WEISER. (Introduced by N. Paul Hudson.)
From the Department of Bacteriology, Ohio State University, Columbus.

The biological interest in deuterium oxide or heavy water cannot be overlooked because all living cells require water for growth. Barnes[1] reported that *Spirogyra* showed some unusual and interesting effects when grown in low concentrations of deuterium oxide and that these concentrations had a stimulating effect on the growth of *Euglena.* Harvey and Taylor[2] recorded that when luminous bacteria were incubated in tubes containing mixtures of heavy water and ordinary water, the amount of oxygen used by the bacteria was in proportion to the percentage of heavy water in the mixture. Also the luminescence of the bacteria was diminished when exposed to low concentrations of heavy water. Richards[3] observed the growth of *Saccharomyces cerevisiæ* in weak concentrations of deuterium oxide which he claimed accelerated growth and development, as opposed to its lethal effect in higher concentrations.

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† The authors wish to acknowledge the technical assistance of Miss Louise Peebles.

‡ After this paper went to press our attention was called to the paper by Rosenthal in *Public Health Reports,* 1937, **52**, 48, which reported results similar to ours.

[1] Barnes, T. C., *Science,* 1934, **79**, 370.

[2] Harvey, E. N., and Taylor, G. W., *Science News Letter,* 1934, **25**, 200.

[3] Richards, O. W., *Am. J. Botany,* 1933, **20**, 679.

In order to obtain data on the reaction of other microbiologic forms, we conducted experiments on the growth and morphology of 8 strains of lactobacilli exposed to various concentrations of this water-compound. We are obligated to Professor H. V. Moyer of the Department of Chemistry for the deuterium oxide.

The heavy water was mixed with Difco dehydrated whey broth so that the broth contained concentrations of 0.13, 0.7, and 5.0% deuterium oxide. The control broth was prepared with distilled water which was devoid of all deuterium oxide. The various lots of broth, in 10 cc. portions, were sterilized in sealed glass tubes at 15 pounds pressure for 20 minutes.

In studying the effect of deuterium oxide, 4 cultures each of *L. acidophilus* and *L. bulgaricus* were used. Each culture was inoculated into the various lots of experimental medium, using 0.1 cc. of an actively growing broth culture for each tube. The cultures were incubated at 37°C. At 12-hour intervals for a 60-hour period, 1 cc. was withdrawn from each tube and pour plates were prepared with tomato-juice agar, using dilutions of 1:1000, 1:10:000 and 1:100,000 of the cultures. After incubation at 37°C. for 48 hours, the plates were examined and the colonies counted.

Examination disclosed that the various concentrations of deuterium oxide in whey broth did not have any appreciable effect on the rate of multiplication of the 8 strains of lactobacilli. The colonies on the plates were examined under low power and failed to show any difference in size or general appearance. The colonies on the plates implanted from the lower dilutions of deuterium-oxide broth were small while those on the higher dilution-plates were somewhat larger, but the differences in the sizes of the colonies were comparable to those in the control medium.

An attempt was made to study the effect of deuterium oxide on the morphology of the 8 lactobacillus cultures, using the same periods of incubation and concentrations of deuterium oxide in broth. Gram-stained preparations did not reveal any remarkable morphologic differences from the control cultures. However, at the end of the 40-hour interval, 3 of the cultures of *L. acidophilus* showed marked granulation and were gram-negative in the 5.0% concentration of deuterium-oxide broth; at the end of the 60-hour incubation period, this granulation was not evident.

The results show that the various concentrations of deuterium oxide in whey broth produced no significant effect on the growth or morphology of the bacteria studied.

9151

Action of Testosterone on Lactation.*

J. M. Robson. (Introduced by A. J. Clark.)

From the Department of Pharmacology, University of Edinburgh.

An inhibitory effect of crystalline oestrone on lactation in mice has been demonstrated by de Jongh[1] and by Robson[2]; inhibition of milk secretion in mice has also been produced by the injection of gonadotropic hormone from pregnancy urine (Enzmann and Pincus.[3])

The present experiments were performed with crystalline preparations of "male hormone", *viz.*, testosterone, testosterone propionate and androsterone. . (For the supply of these compounds I am greatly indebted to Messrs. Ciba, Ltd.)

The experiments were performed on mice; a number of animals were injected daily with 0.1 mg. of testosterone propionate in oil, the first injection being given on the day of parturition, or on the morning after parturition when this occurred during the night. The injections were continued for 20 days or until the death of the litter. In all cases there was an appreciable inhibitory effect on the growth of the litters and all but one died from lack of food before reaching the age of 3 weeks. Some inhibition was also produced by injecting the mothers with 0.05 mg. of testosterone propionate per day. Administration of 0.1 or 0.2 mg. of androsterone in oil twice a day had no such effect.

In some experiments testosterone was given as a fine suspension in 10% alcohol over a period of 72 hours or more; 0.05 mg. in 0.05 cc. was injected every 2 hours during the day and twice that amount was given at midnight and at 4 a. m. These injections rapidly brought lactation to an end and the litters died 3-4 days after birth. Suckling and maternal care were not inhibited. Similar injections of androsterone produced only a slight inhibition of growth and the administration of the solvent (*i. e.*, 10% alcohol) had no effect. Further investigations with other compounds are in progress.

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* The expenses of this investigation have been defrayed by a grant from the Medical Research Council.

[1] de Jongh, S. E., *Acta Brevia Neerland. Physiol.*, 1933, **3**, 52.

[2] Robson, J. M., *Quart. J. Exp. Physiol.*, 1935, **24**, 337.

[3] Enzmann, E. V., and Pincus, G., *Am. J. Physiol.*, 1933, **103**, 30.

9152

A Sealed-In Micro Glass Electrode.

Grace E. Pickford. (Introduced by Ross G. Harrison.)
From the Osborn Zoölogical Laboratory, Yale University.

A sealed-in glass electrode essentially similar to that described by Michaelis[1] but of much smaller capacity can readily be adapted for pH measurements of biological fluids when only rather small volumes are available and under conditions which exclude contact of the sample with air.

The design is shown in the accompanying diagram (Fig. 1); the inner tube of Corning O15 glass may be of capillary dimensions but care must be taken to minimize the capacity of slight but unavoidable dilations at the two ends where this capillary is sealed, through heavy walled graded glass, to the outer jacket. When this unit, the capillary electrode within its jacket, has been tested and found free of electrical leaks or other defects it is attached permanently to a stopcock of the type described by Stadie, O'Brien and Laug.[2] For testing it is convenient to connect it temporarily with rubber tubing to a spare stopcock of the same type. Redescription of this stopcock is not necessary but two points should be mentioned: (1) in order that the electrode may be permanently attached, as at X in the diagram, the stopcock must be made of soft glass similar to that of the electrode jacket. (2) In order to reduce the volume of the test sample to a minimum the diagonal bore for filling must be as fine as possible and without unnecessary widening at the ends. This bore has a diameter of 1 mm. in the electrode assemblies in use by the author; the bore of the filling and connecting tube is about 0.5 mm.

The method of filling the electrode depends, of course, on the technique and requirements of sampling. In an investigation of the digestive juice and blood of tarantulae it has been convenient to withdraw the sample directly from the animal into a 1 ml. Luer syringe (the short "Insulin" type is preferable). The needle is then removed and a short piece of haemocytometer tubing (3 mm. internal, 5 mm. external diameter) is fitted over the nozzle. The filling inlet on the stopcock is provided with a cup which exactly fits the nozzle when such a piece of rubber tubing has been slipped over it (see diagram). In order to avoid contact of the sample with air the syringe may be first filled with freshly boiled distilled water which is then ejected so as to leave water rather than air in the bore of the needle and nozzle. The total volume of such remaining water is about 0.05 ml. This about equals the capacity of the smallest electrode at present in use so that very small samples are diluted with about an equal volume of water. When larger volumes are available it is of course better, if possible, to fill the syringe with a preliminary rinse sample so that there shall be no subsequent dilution of the test sample.

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[1] Michaelis, L., *Science,* 1936, **83**, 213.

[2] Stadie, W. C., O'Brien, H., and Laug, E. P., *J. Biol. Chem.,* 1931, **91**, 243.

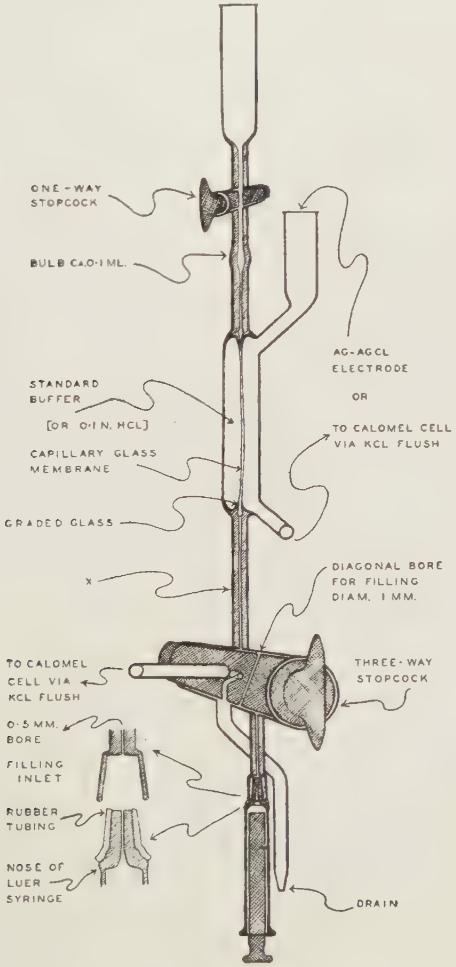

FIG. 1.

The electrode is calibrated with a standard buffer, washed and then dried with alcohol and ether; I can confirm the findings of

Stadie, *et al.,* that this treatment has a negligible effect on the asymmetry potential. The sample is then introduced slowly from the syringe into the electrode. Preferably, when more than the minimum volume is available, the first part of the sample is passed over into a small dilation above the electrode, thus eliminating that part which came into contact with the atmosphere. Both upper and lower stopcocks are then closed, after which the sample may be allowed to remain in the electrode for a considerable time without deterioration. Before making a measurement the lower stopcock is rotated clockwise so as first to flush saturated KCl through the groove and right angle bore—the position shown in the diagram—and then to make liquid junction between the sample and the saturated calomel half cell on the left. If the sample is required for other purposes it is not necessary to put it in full contact with the KCl provided the inner race of the stopcock is wetted with this solution. Under these conditions the sample is scarcely contaminated and may be withdrawn into the syringe after measurement and used, for example, in digestion experiments.

After use the electrode is washed with dilute salt solution and kept filled with distilled water. The outer jacket may be filled with N/10 HCl and provided with a permanent silver-silver chloride electrode, as recommended by Michaelis. In one assembly in use by the author the jacket is kept filled with distilled water; just before use this is replaced with M/20 K H Phthalate or some other standard buffer solution and connected by a lower outlet with a second KCl reservoir and calomel half cell on the right. The 2 calomel half cells agree within 0.1 mv. This arrangement permits immediate determination of changes in asymmetry potential, uncomplicated by other factors; on the other hand it is somewhat more troublesome and quite unnecessary for routine measurements of pH.

Two of these micro electrodes, one with a total capacity of 0.25 ml. and the other of 0.06 ml., have been in use in this laboratory for periods of nearly a year and 6 months respectively. A third sealed-in electrode of larger capacity, similar to that described by Michaelis except that it is filled by suction from above, is provided with an upper stopcock and is connected to the lower stopcock by an interchangeable grinding, is also in constant use and has proved most satisfactory. The asymmetry potentials were at first high but have since dropped almost to zero. The resistance is high, ca. 219 and 292 megohms for the 2 micro electrodes, ca. 146 megohms for the larger electrode, but this is of little importance since the assembly is used in conjunction with a Pliotron tube amplifier of the type described by Du Bridge and Brown.[3] The circuit includes a Leeds & Northrop type K potentiometer and type R galvanometer; 1 mv. gives a deflection of about 4 mm. on the scale at one metre. No erratic behavior has been encountered on the part of these electrodes; there may be slight hysteresis after changing from one buffer solution to another of widely different pH but the effect wears off within a few minutes after careful washing. With all ordinarily well buffered solutions, including blood samples from spiders, we regularly obtain steady readings within ±0.2 mv. or better. The value of the constant RT nF became practically normal after the first few weeks of use.

Although the initial outlay may be greater than for many designs hitherto published, the assembly here described is so convenient that it seems probable that it might find a use in other laboratories. An assistant can be trained to make routine measurements without difficulty. The amplifier is simple to operate provided proper shielding requirements are observed, in particular the grid lead should be thoroughly shielded and insulated. As pointed out by Hill[4] the high input resistance of the Pliotron eliminates the need for making fragile glass membranes of very low resistance.

The electrodes described above were made by the Macalister Bicknell Company of Cambridge and New Haven. The amplifier was built by Dr. A. Petrunkevitch of this department, who has designed a very convenient non-capacity switch (unpublished). We are indebted to Mr. D. G. Brubaker and the Sloane Physics Laboratory of Yale University for measuring the constants of the Pliotron used and also for assistance in determining the resistance of the electrodes.

9153

Preparation and Properties of a Specific Polysaccharide from a Strain of *Vibrio Cholerae*.*

D. L. SHRIVASTAVA AND S. C. SEAL. (Introduced by R. W. Linton.)

From the Cholera Inquiry, Indian Research Fund Association, All-India Institute of Hygiene and Public Health, Calcutta.

After a number of trials the method described below gave satisfactory results in the preparation of a specific polysaccharide of the strain known as Inaba variant, which belongs to Group VI in the chemical classification of the vibrios (Linton, Shrivastava and Mitra[1]). The method is based on that of Heidelberger, Kendall and Scherp.[2]

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[3] Du Bridge, L. A., and Brown, H., *Rev. Sci. Instr.*, 1933, **4**, 532.

[4] Hill, S. E., *Science*, 1931, **73**, 529.

* This work was done under the auspices of the Indian Research Fund Association and is published by the permission of the Secretary.

To the 72-hour growth of Inaba variant in 21 liters of 1% peptone water (pH 8.0), phenol was added to make a final concentration of 0.5% and the mixture allowed to stand for an hour. The bacterial growth was then removed in a Sharples supercentrifuge. The peptone water was neutralized with acetic acid and concentrated *in vacuo* to 2 liters. To the concentrate 60 gm. of sodium acetate and 12 cc. of glacial acetic acid were added and the mixture precipitated with 2 volumes of absolute alcohol, or if necessary for complete precipitation, 3 volumes. Larger amounts of alcohol were found to bring down peptone, inorganic salts, etc., in quantities which greatly interfered with the subsequent purification. The precipitate was taken up in 150 cc. of water, 3 or 4 cc. of acetic acid and 10 cc. of saturated sodium acetate solution, and centrifuged; if the material thrown down gave only a faintly Molisch positive reaction it was discarded. If it gave a strong reaction, it was again dissolved in water, treated as above, and the 2 centrifuged solutions were mixed.

The solution was precipitated with a minimum amount of alcohol, one-half volume usually being found sufficient. This precipitate was taken off and further addition of alcohol to the supernatant usually yielded only a very small precipitate which could be neglected. The above process was repeated and the final precipitate was taken up in a minimum amount of saturated sodium acetate (20 cc.) and about 10 cc. of acetic acid, centrifuged, and the supernatant precipitated with chilled acetic acid. Usually 15 to 20 volumes were required to give a flocculent precipitate. After centrifuging, the precipitate was taken up in water and centrifuged, and the biuret-positive insoluble matter was discarded. The solution was tested for biuret, Molisch reaction, and serological activity with homologous antiserum. If it was still biuret-positive it was shaken with chloroform to remove the last trace of protein. For this purpose, the solution was made up to 200 cc. with water, 20 cc. of chloroform were added, together with 4 cc. of amyl alcohol, 5 g. of sodium acetate and 4 cc. of acetic acid. It was then shaken mechanically for about half an hour and centrifuged. The topmost layer contained the polysaccharide in solution and was decanted carefully and shaken again with chloroform and amyl alcohol until the amount of the denatured protein layer became extremely small. After the final centrifuging the top layer was filtered and the filtrate precipitated with 1 to 2 volumes of alcohol.

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[1] Linton, R. W., Shrivastava, D. L., and Mitra, B. N., *Indian J. Med. Res.*, 1935, **22**, 633.

[2] Heidelberger, M., Kendall, F., and Scherp, H. W., *J. Exp. Med.*, 1936, **64**, 559.

The precipitate was taken up in about 20 cc. of saturated sodium acetate, and an appreciable amount taken for the biuret test. To the acetate solution 3 cc. of acetic acid were added and the whole was made up to 100 cc. with water and precipitated again with one volume of absolute alcohol. At this stage the solution of the precipitate was biuret-negative, and 3 or 4 alcohol precipitations in the presence of sodium acetate and acetic acid at approximately pH 4.0 were carried out, centrifuging the water solution each time to free it from any extraneous matter. The final precipitate was taken up in water, and after centrifuging, redistilled alcohol was added until opalescence or a slight precipitate appeared. The minimum amount of sodium acetate necessary for a flocculum to appear was then added. This precipitate was washed twice with redistilled alcohol, followed by acetone and quickly filtered on hardened filter paper and dried *in vacuo*. Yield: 0.297 gm.

The dried substance was white in color and extremely fibrous, resembling fibers of dried filter paper pulp. It was soluble in water with difficulty, and gave a solution of high viscosity even at a concentration of less than 3 mg. per cc. It was free from glycogen and phosphorus.

The Molisch test was positive at a dilution of 1:6,000,000. The material had a nitrogen content of 2.62%, 7.8% of ash (as Na_2O), and a specific rotation of +58.0°. Hydrolyzed in the boiling water bath with 3% H_2SO_4 (by volume), it gave the following reductions:

Time Interval. Hr.	% of Reducing Substance as Glucose
1	22.2
2	33.0
3	36.6
4	57.1
5	62.2
6	57.9

Beginning at the 4-hour period, the hydrolysate was inactive with homologous antiserum. The sudden rise in reducing power between the 3d and 4th hours appeared to indicate the presence of a second substance in the solution whose rate of hydrolysis differed from that which hydrolyzed first; this observation recalled earlier work, in which certain preparations showed the presence of a difficultly hydrolyzable aldobionic acid (Linton and Shrivastava[3]). The phenylosazone obtained from the hydrolysate had a melting point of 206°; mixed with known glucosazone the melting point was unchanged. Attempts were made to isolate other reducing substances in the hydrolysate, but up to the present they have not been successful. Earlier work[1] on the glucose-containing type of polysaccharide was also unable to bring to light any other constituent than glucose, in contrast to the arabinose- and galactose-containing types of vibrio polysaccharide in which an aldobionic acid is present.

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[3] Linton, R. W., and Shrivastava, D. L., *Indian J. Med. Res.*, 1933, **21**, 91.

With homologous antiserum, the polysaccharide reacted strongly. A zone phenomenon was present between dilutions of 1:4,800 and 1:150,000, but beginning at the latter dilution a gradually increasing floccular reaction was evident up to 1:12,000,000, after which it disappeared. The maximum reaction lay at about 1:2,500,000. Readings were taken after the tubes had been for 2 hours at 52°C. and finally after having been kept overnight in the ice-chamber.

The specificity of the polysaccharide was also tested by the use of antisera representative of each of the 6 groups into which the vibrios as a whole are divisible on the basis of their chemical constitution (Linton[4]; Linton and Mitra[5]; Linton, Shrivastava and Mitra[1]).

TABLE I.

Precipitin reactions of Inaba Variant (Group VI) Polysaccharide and Inaba (Group I) Polysaccharide with antisera derived from members of the six vibrio groups.

Group	Antisera (1:10)	Inaba Variant Polysaccharide	Inaba Polysaccharide
I	Inaba	0	1:400,000
	Kasauli 11	0	1:400,000
	10404	0	1:25,000
	Rangoon Smooth	0	1:100,000
II	653 Original	0	0
	984 Habigunj	0	0
	3065 Habigunj	0	0
III	W880	0	0
	653 (11-D)	0	0
IV	El Tor	0	0
	676	0	0
	Nanking 32/109	0	0
V	El Tor 34/D/19	0	0
	Doorenbos 34/11 (El Tor)	0	0
	6380 of 14/1/35	0	0
VI	Ogawa	1:2,500,000	0
	Inaba Variant	1:12,000,000	0

All controls were negative.

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[4] Linton, R. W., *Bull. Off. Internat. d'Hyg. Publique*, 1935, **27**, No. 6.

[5] Linton, R. W., and Mitra, B. N., PROC. SOC. EXP. BIOL. AND MED., 1934, **32**, 468.

The results are given in Table I, which also includes a similar test with the polysaccharide of a Group I strain, Inaba. This polysaccharide had been prepared in one of the earlier attempts to perfect the method, and hence does not react highly with its homologous antiserum. It is, however, specific for the Group I strains.

Although the number of strains studied is few as yet, it is evident that the polysaccharides give group reactions so far as the data go. It is further interesting to note that the 3 El Tor antisera are not reactive with the Inaba polysaccharide. El Tor strains cannot be differentiated from cholera strains by "O" agglutination (Gardner and Venkatraman[6]); or by the vibrio polysaccharides prepared by Bruce White,[7] but they appear to be differentiated serologically by the polysaccharide used in the present experiments.

A further example may be given to show the relationship which is being demonstrated between serological reactivity and chemical structure in the vibrios. In April, 1935, chemical analysis of strain 1200 which had been isolated from a carrier in the previous December, showed that the organism belonged to Group VI, *i. e.,* its polysaccharide was of the glucose-containing type, similar to that of Inaba variant. A second analysis of the polysaccharide fraction carried out in May, 1936, indicated the presence in the hydrolysate of a large amount of galactose, accompanied by some glucose. When the antiserum against this strain was tested with Inaba variant polysaccharide in January, 1937, no reaction was obtained, and accordingly a third analysis of the polysaccharide was done. This revealed the presence of galactose in pure form, and a subsequent test of the antiserum with Inaba polysaccharide (which is of the galactose-containing type) showed a strong reaction. The shift in chemical structure was thus indicated by the precipitin reaction of the polysaccharide. It would appear from this experience that serological methods may enable us to follow the changes in chemical structure which occur in the vibrio group, and which have previously been described at length.[1, 4]

Polysaccharides from strains representative of all the chemical groups are now in course of preparation.

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[6] Gardner, A. D., and Venkatraman, K., *J. Hyg.,* 1935, **35**, 262.

[7] Bruce White, P., *Brit. J. Exp. Path.,* 1936, **17**, 229.

9154

Experimental Menstruation in the Monkey after Removal of Ovaries and Adrenals.

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Menstruation is preceded by vasoconstriction of endometrial vessels and hemostasis which begins 6 to 12 hours before menstruation and may persist throughout the first day. This observation was made by Markee[1] in living endometrium of the monkey transplanted to the anterior chamber of the eye. Bartelmez[2] had previously emphasized the part played by the spiral arteries in menstruation and the recent study of these vessels in the monkey by Daron[3] has focussed attention on this aspect of the menstrual cycle.

It has long been known that double ovariectomy is followed in a few days by menses and then by disappearance of menstruation and atrophy of the uterus. Experimental menstrual cycles can be induced in the ovariectomized monkey by injections of ovarian follicular hormone (theelin, oestrin). This hormone produces an effect upon the uterus in 6 to 10 days which is followed by menstruation (Allen,[4, 5] Morrell, Powers, Varley and de Frates,[6] and others). Eighty to 150 rat units of theelin in 6 to 10 days is the menstrual threshold for adults, larger doses are required for immature monkeys, but 350 rat units in 10 days will produce menstruation in infantile monkeys of 1500 gm. body weight (Allen, Diddle, Burford and Gardner[7]). The thyroid gland can be completely removed without affecting the ovarian hormone threshold necessary to produce experimental menstruation (Burford, Allen, Diddle[8]).

Assuming that premenstrual vasoconstriction and hemostasis may be essential in producing the hemorrhage and desquamation which follow, the secretion of the adrenal medulla might be a factor con-

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* This work was supported by a grant from the Committee for Research in Problems of Sex of the National Research Council.

[1] Markee, J. E., *Anat. Rec.*, 1933, **55**, 66.

[2] Bartelmez, G. W., *Carnegie Inst. Wash.* No. 443, *Contrib. to Embryol.*, **24**, 141-186.

[3] Daron, G. H., *Am. J. Anat.*, 1936, **58**, 349.

[4] Allen, E., *Anat. Rec.*, 1926, **32**, 226.

[5] Allen, E., Contrib. to Embryol., Carnegie Inst. of Washington, 1927, **19**, 1.

[6] Morrell, J. A., Powers, H. H., Varley, J. R., and de Frates, J., *Endocrinology*, 1930, **14**, 174.

[7] Allen, E., Diddle, A. W., Burford, T. H., and Gardner, W. U., *Am. J. Physiol.*, 1936, **117**, 381.

[8] Burford, T. H., Allen, E., and Diddle, A. W., 1936, **20**, 635.

tributing to the onset of menstruation. The present preliminary experiments were made to test the effect of removal of all suprarenal medullary tissue on experimental menstruation induced by theelin† in ovariectomized monkeys.

Adrenalectomy was done in 2 stages, several weeks elapsing between removal of right and left glands. On the day following removal of the last suprarenal, injection of cortical extract was begun. This extract was prepared and standardized by Dr. Swingle. Doses ranging from 1.7 to 3.0 cc. given twice daily were adequate to prevent adrenal cortical deficiency.

Theelin was injected twice daily for 10 days, 50 r.u. per day in monkey No. 50, and 60 r.u. in monkey No. 62. A marked increase in the redness of the "sexual skin" was noted. A latent period of 5 days followed the last injection and then menstruation began in both animals. The flow was profuse on the second and third days and menstruation lasted for 5 days in monkey No. 50 and for 7 days in monkey No. 62.

There was enough cortical extract to last for 21 days. The monkeys were then observed for onset of cortical deficiency. During this period monkey No. 50 was injected with theelin, 60 r.u. daily for 11½ days. There followed a latent period of 4 days and then 4 days of menses.

Thus 3 experimental menstrual periods were obtained in 2 ovariectomized monkeys following doses of 500, 600 and 690 r.u. of theelin and after removal of all adrenal medulla. These menses differed in no way from the usual course of events previously observed in ovariectomized animals with intact suprarenals under similar hormone treatment.

Signs of adrenal insufficiency appeared as expected in monkey No. 62 but not in No. 50, and at autopsy a nodule of cortical tissue one cm. in diameter was found adherent to the vena cava in the right suprarenal region in the latter. Since this contained no medullary tissue, it had little bearing on the main objective of the experiment.

Summary. Both ovaries and suprarenals were removed from 2 monkeys which were then injected with cortical extract (Swingle). Three experimental menstrual periods were then induced by injections of theelin—total doses ranging from 500 to 690 r.u. in 10 to 11½ days. Latent periods of 4 to 5 days followed the last injections and then menstrual periods of 4 to 7 days. The adrenal medulla is not essential to experimental menstrual periods induced by theelin.

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† The theelin for this experiment was furnished by Parke, Davis & Co., through the courtesy of Dr. Oliver Kamm.

9155 P

Isolation and Properties of the Factor Responsible for Increased Capillary Permeability in Inflammation.*

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The previous studies of the writer have indicated the presence of a factor in various types of exudates capable of promptly increasing the permeability of the capillary wall.[1, 2] The liberation of this substance by injured tissue and its consequent recovery in the exudate has offered a reasonable explanation for the main mechanism of increased plasma filtration into an inflamed area. The factor exhibited none of the properties of histamine or of its presumably closely related H-substance; thereby rendering it difficult to accept the views of Sir Thomas Lewis on the subject.[2, 3] The active substance was shown to be dialyzable and was recovered as a crystalline protein-free material.

An attempt was made to isolate the active factor relatively free of any gross impurities. The method adopted for extracting and recovering the crystalline substance was as follows: The exudate containing the factor, is as a rule obtained in abundance, as described in previous communications, from dogs after intrapleural injection with turpentine.[4, 2] The exudate, separated of its cell content by centrifugalization, is treated with an equal volume of pyridine. The precipitated mixture is placed in a stirring apparatus for several hours and then centrifuged. The supernatant fluid contains the active fraction. It is treated with 3 times its volume of acetone. A heavy white flocculating mass precipitates out which is discarded after centrifugalization. The active factor seems to be contained exclusively in the supernatant fluid. This is evaporated to dryness *in vacuo* at a temperature ranging between 40° to 50°C. This dried acetone residue may contain some brownish tarry material interspersed in a mass of white crystalline material. In this material there may be discerned numerous "branched" or "notch-like" crystals. These crystals are characterized by the presence of a main shaft with short

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* Aided by grants from the Milton Fund, Harvard University; the Wellington Memorial Research Fund, and the Permanent Charity Fund of The Harvard Medical School; and by grants from the Committee for Scientific Research and from the Council of Pharmacy and Chemistry, American Medical Association.

[1] Menkin, V., PROC. SOC. EXP. BIOL. AND MED., 1936, **34**, 570.

[2] Menkin, V., *J. Exp. Med.*, 1936, **64**, 485.

[3] Lewis, T., and Grant, R. T., *Heart*, 1924, **11**, 209.

[4] Menkin, V., *Am. J. Path.*, 1934, **10**, 193.

lateral projections on either side. The residue is only partially soluble in water. Further purification is attained by filtering the aqueous suspension and evaporating *in vacuo* the filtrate. The resulting crystalline material is characterized by practically only one type of structure. These are the characteristic "branched" or sometimes "cross-like" crystals having essentially the same pattern as the crystals observed in the acetone residual fraction. There may be a moderate amount of tarry material admixed to the crystalline residue recovered after drying the acetone fraction. Some of this may be removed by shaking the aqueous suspension with butyl alcohol and subsequently by separating off the clear layer. Evaporation of this fraction yields the active crystalline substance which manifests essentially a similar structural pattern as the crystals recovered from the aqueous or the acetone fractions. Finally, the supernatant fluid after precipitation with acetone may be dialyzed for several hours against distilled water. Evaporation of both the dialyzed and the dialyzate fractions reveals the presence of the characteristic crystals. This indicates that the substance diffuses readily through a cellophane membrane.

The typical crystals recovered, as described above, are extremely active in increasing capillary permeability. This is indicated by a method previously described.[2] The crystals are readily dissolved in distilled water or saline. The solution is injected intracutaneously in the dermis of the abdomen of a normal rabbit. Trypan blue is immediately injected intravenously. This dye accumulates abundantly within several minutes in the treated cutaneous area, indicating thus a local increase in the filtration capacity of the small vessels, precisely as is the state of affairs when the untreated cell-free exudate is used. Distilled water or saline induce no accumulation of dye. Pyridine and acetone mixtures were evaporated to dryness to control for the reagents employed in isolating the active factor. None of the typical crystals were recovered and the dried mixture, treated with distilled water, failed to induce the passage of dye from the blood stream when injected intracutaneously. The isolated typical crystalline substance seems therefore to represent the active factor present in exudates and capable *per se* of increasing capillary permeability. Similarly to the untreated exudate and unlike histamine, this crystalline substance depresses the tonus of the isolated strip of guinea pig intestine.

When normal blood serum containing no trace of hemolyzed red blood corpuscles is treated precisely as an exudate with pyridine and acetone, a superficially similarly appearing crystalline substance, but apparently in reduced concentration, may be recovered. The crystals, however, seem to be more soluble in water than those isolated from an exudate. The solution of crystals obtained from blood serum are wholly or practically inactive when injected intracutaneously. Trypan blue barely accumulates in such treated skin areas. This had likewise been found to be true of the unfractionated blood serum.[2] On the other hand, blood serum, exhibiting evidence of considerable amounts of hemolyzed red blood corpuscles (or in some cases apparently extensive lipemia), when extracted as outlined above, tends to yield typical crystals which are indistinguishable from those recovered from exudates. Such crystalline material may display definite potency in the skin as evidenced by pronounced accumulation of dye from the circulating blood. This suggests that the active factor is probably liberated from injured or disintegrated tissue cells.

The active crystals recovered from exudates are water soluble. They dissolve in acetone and alcohol; but are apparently insoluble in ether or absolute alcohol. The material is heat stable. Its solution yields a negative Biuret test for proteins. The Molisch and Fehling tests indicate that the material is not a carbohydrate. Unlike histamine, which has a melting point of about 130°C., it shows no sharp melting point. At about 175°C. it chars and at 265°C. to 298°C. it still has not melted. The available evidence from proteolytic studies of total protein nitrogen and amino acid nitrogen of exudates show a reciprocal relationship when compared with their level in blood serum. The average protein nitrogen of dog blood serum was found to be 6.1 gm./100 cc. compared with a value of 4.75 gm./100 cc. in exudates. On the other hand the average figure of serum amino acid nitrogen was found to be 5.55 mg./100 cc. as compared with 8.04 mg./100 cc. in exudates. The increase in the amino acid nitrogen of exudates was correlated by the activity of the latter on increasing the permeability of capillaries. This suggests that the active substance may well be an intermediary product of protein catabolism. Its nitrogenous content averages 2.3%. The pH of its aqueous solution may be slightly acid (6.5) or slightly alkaline to phenol red. It is precipitated out by saturated $(NH_4)_2SO_4$. It is readily dialyzable. It appears unlikely on the basis of accumulated observations that it is either a proteose, a peptone, or an amino acid. The present available data seem to indicate that this active substance probably belongs to the group of polypeptides. Further studies are being conducted in an attempt to free completely of all impurities the active substance and to identify it chemically.

It is important to note that the crystalline material is evidently capable of initiating the sequences of the inflammatory reaction. Its intracutaneous injection is not merely followed by a prompt increase in capillary permeability; but within a surprisingly short interval of 25 to 30 minutes there is considerable evidence of leucocytic migration into the treated areas. The small vessels are packed with polymorphonuclears closely adhering to the endothelial wall in the act of migrating into the extra-capillary spaces which reveal already moderate infiltration of these cells. Furthermore, the substance is positively chemotactic when placed in capillary glass tubes sealed at one end and introduced into an inflamed peritoneal cavity, the tubes soon become filled with leucocytes. The inactive crystalline material obtained from blood serum and injected intracutaneously, distilled water, or saline are all totally devoid of inducing such prompt accumulation and migration of leucocytes through the endothelial wall. Turpentine, which in the skin causes a rapid increase in capillary filtration as indicated by the pronounced seepage of dye in the affected area, is equally incapable of exhibiting as rapid a migration of leucocytes as the active crystalline material. The implications of these observations are obvious. The studies are being continued and will be reported *in extenso* elsewhere.

Conclusions. For the sake of convenience the name *leukotaxine* is tentatively proposed for this active crystalline nitrogenous substance which is evidently released by injured tissue and is readily recovered in inflammatory exudates. "Leukotaxine" *per se* appears to be capable of rapidly initiating the usual sequences of the inflammatory reaction first by inducing a prompt increase in capillary permeability and secondly by causing an extremely rapid aggregation and migration of leucocytes through the endothelial wall.

9156

Cane Molasses versus Beet Molasses as a Source of Vitamin B_6 and Lactoflavin.

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Regardless of the fact that Nelson, Heller and Fulmer[1] published experimental evidence concerning the value of cane molasses as a source of the whole vitamin B complex, molasses is still generally looked upon as a qualitatively inert food constituent.

In 1935 the experimental data of Nelson, Heller and Fulmer received substantial confirmation. Birch, György and Harris[2] showed that cane molasses is very rich in vitamin B_6. As molasses is one of the major components of the diet of pellagrins in the cotton districts of the United States, the high vitamin B_6 potency of molasses indicates that vitamin B_6 has no antipellagra action for human beings.

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[1] Nelson, V. E., Heller, V. G., and Fulmer, E. I., *Indust. and Engin. Chem.*, 1925, **17**, 199.

Furthermore, Jukes and Lepkovsky[3] recently have found in cane molasses a fairly good source of the preventive factor for chicken pellagra, the so-called "filtrate" factor; it therefore seems unlikely that lack of the chicken pellagra factor would be responsible for the occurrence of human pellagra.

Nelson, Heller and Fulmer measured the whole vitamin B complex, its composite nature being unknown at that time, and they established the fact that cane molasses is far superior to beet molasses as a source of vitamin B. I have repeated these experiments, using

TABLE I.

Average values for distribution of lactoflavin and vitamin B_6 in molasses.

Molasses Sample Fed to Rats	Amt. Given Daily	Tests for Lactoflavin (Vitamins B_1 and B_6 provided):	Tests for Vitamin B_6 (Vitamin B_1 and Lactoflavin provided):	
		Aver. Weekly Increase in Wt. of Rats	Aver. Weekly Increase in Wt. of Rats	Healing of Specific Dermatitis
	gm.	gm.	gm.	
Cane molasses I, blackstrap (Louisiana)	0.075	—	3	No
	0.125	—	5	Yes
	0.25	—	6.5	"
	0.5	—	—	—
	0.75	2.5	—	—
	1.5	7.5	—	—
Beet molasses (Germany)	0.25	—	0	No
	0.5	—	–1	"
	0.75	0	1	"
	1.0	—	–1	"
	1.5	–4	—	—
Beet molasses (Michigan)	0.75	—	–1	No
	1.5	—	0	"
Syrugold	0.25	—	–2	"
	0.5	—	2	"
	0.75	—	0	"
	1.5	–1	—	—
Karo (blue label)	0.5	—	–2	No
	1.0	—	2	"
	2.0	—	3	Inconstant
	2.5	1.5	2.5	Yes
	5.0	–2	—	—

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[2] Birch, T. W., György, P., and Harris, L. J., *Biochem. J.*, 1935, **29**, 2830.

[3] Jukes, T. H., and Lepkovsky, S., *J. Biol. Chem.*, 1936, **114**, 117.

my method for the separate determination of lactoflavin and vitamin B_6.[4] I have also tested Karo (blue label), which contains a molasses preparation, and Syrugold, a refined cane molasses preparation (National Sugar Refining Co.), for potency of vitamin B_6 and lactoflavin. The results are recorded in Table I.

In addition to the sample of blackstrap molasses given in Table I, I tested 8 other samples of crude cane molasses for their vitamin B_6 content. In 7 samples, from Louisiana, the vitamin B_6 titer varied between 0.1 and 0.3 gm. per rat per day (in two it was 0.1 gm., in three 0.15 gm. and in two 0.3 gm.); in the eighth sample, of unknown provenience, the titer was 0.4 gm. per rat per day. In a specimen of commercial blackstrap treacle put up by Fowler (England), the rat-day dose of vitamin B_6 was found[2] in 0.25 gm. Beet molasses samples from Germany and from Michigan were both devoid of vitamin B_6 (see Table I).

The Karo sample recorded in Table I showed B_6 activity at the daily dose per rat of 2 to 2.5 gm., whereas in 2 other samples of blue label Karo I failed to detect any curative action for deficiency of vitamin B_6. The implications of this finding in connection with questions of infant feeding will be discussed elsewhere.[5]

Conclusions. Crude cane molasses is a good source of vitamin B_6, the B_6 titer varying from 0.1 to 0.4 gm. per rat per day, with an average of 0.15 gm. Crude cane molasses also contains small amounts of lactoflavin. Beet molasses is practically devoid of both vitamin B_6 and lactoflavin.

9157 P

Influence of Carbon Dioxide upon Blood Pressure Reaction to Oxygen Deficiency.

E. H. LAMBERT AND E. GELLHORN.

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Gellhorn[1] showed recently that CO_2 either completely offsets or greatly diminishes the effects of oxygen deficiency on various sensory and psychic phenomena. This effect may be thought to be due to the effect of CO_2 on respiration whereby the acapnia associated with the increased breathing due to oxygen deficiency is eliminated.[2] The oxygen tension in the alveolar air is increased and the oxygenation of the tissues is improved due to the right shift in the oxygen and dissociation curve. It is, however, not improbable that in the very striking restoration of function due to the administration of small amounts of CO_2 circulatory adjustments may be involved also. This is shown by studies of Henderson and collaborators,[3] who found an increase in muscular tonus to occur after the administration of small amounts of CO_2 which by increasing the venous return to the heart improved circulation. Gellhorn[4] found, in his studies on humans, that when the subjects were in erect posture the systolic blood pressure fell considerably, whereas it was maintained at a normal level or remained slightly elevated during the experiment when 3% CO_2 was inhaled simultaneously with the oxygen deficient gas mixture.

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[4] György, P., *Biochem. J.*, 1935, **29**, 741.

[5] György, P., in preparation.

[1] Gellhorn, E., *Nature*, 1936, **137**, 700; *Am. J. Physiol.*, 1936, **117**, 75; *Proc. Am. Physiol. Soc.*, 1936, p. 59; Sigma Xi Quarterly, 1937 (June), in press.

A more quantitative study of the effect of CO_2 under the conditions of oxygen deficiency seemed desirable and blood pressure studies were undertaken on dogs anesthetized with sodium amytal or sodium barbital under conditions of normal respiration, and also after the chest was opened and artificial respiration was instituted. The gas mixtures were administered through Douglas bags for 60-90 seconds. Table I gives some typical results from which can be seen that, although the effect of 4% CO_2 is in most experiments negligible, it increases the rise in blood pressure due to the breathing of an oxygen deficient gas mixture more than corresponds to the algebraic sum of the effects of either of the two factors on the blood pressure. This effect could be thought to be due to the removal of

TABLE I.
Rise of blood pressure in dogs due to the inhalation of various O_2 and CO_2 mixtures.

O_2-deficiency		CO_2 in air		CO_2+O_2-deficiency		
O_2-concentration %	Blood pressure in mm. Hg.	CO_2-concentration %	Blood pressure in mm. Hg.	CO_2 concentration %	O_2 concentration %	Blood pressure in mm. Hg.
		I Respiration Intact				
3	+10	4	+2	4	3	+18
5.5	+ 3	4	+1	4	5.5	+ 7
5.5	+ 3	4	+2	4	5.5	+11
		II Artificial Respiration				
5.5	+17	4	+6	4	5.5	+32
5.5	+ 8	4	+2	4	5.5	+15
3.0	+15	4	±0	4	3.0	+27

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[2] Childs, S. B., Hamlin, H., and Henderson, Y., *Nature*, 1935, **135**, 457.

[3] Henderson, Y., Oughterson, A. W., Greenberg, L. A., and Searle, C. F., *Am. J. Physiol.*, 1936, **114**, 261.

[4] Gellhorn, E., *An. Int. Med.*, 1937, **10**, 1267.

the acapnia resulting from the increased respiratory volume occurring under oxygen deficiency. If, however, artificial respiration is instituted, the CO_2 tension in the blood remains constant; and any changes in the efficiency of CO_2 must be attributed to its effect on the circulatory rather than the respiratory system. Under these conditions similar results were obtained. Here again the rise in blood pressure was greater in the oxygen deficient gas mixture containing 4% CO_2 than corresponded to the sum of the rises obtained when either one of the two factors was administered separately. This potentiation of the CO_2 and oxygen deficiency effect on the blood pressure must be considered to be of utmost value in maintaining an adequate circulation through the brain. The results indicate that the beneficial effect of CO_2 in offsetting oxygen deficiency is due not only to its influence on respiration but to augmenting greatly the rise in blood pressure which occurs under the conditions of oxygen deficiency.

Summary. The rise of blood pressure caused by oxygen deficiency is greatly augmented or potentiated by small amounts of CO_2 which in themselves have no effect upon blood pressure. This potentiation is present not only in the animal capable of respiratory adjustments, but also when the minute volume of ventilation is kept constant by means of artificial respiration. These conditions indicate that the circulatory system, *per se,* does play a major part in the adjustment to oxygen deficiency brought about by small amounts of CO_2.

9158

Multiplication of Yellow Fever Virus in the Developing Chick Embryo.

JOHN E. ELMENDORF, JR., AND HUGH H. SMITH. (Introduced by J. H. Bauer.)

From the Laboratories of the International Health Division, Rockefeller Foundation, New York.

Since Woodruff and Goodpasture[1] first discovered that fowl pox virus could be grown on the chorio-allantoic membranes of developing chick embryo in the egg, many workers have reported success in the propagation of other viruses by this method. It was shown[2, 3] that yellow fever virus could be grown *in vitro* in a medium containing chick embryonic tissues. It was considered of interest to determine whether this virus could be maintained in the living embryos. Using the window-technique of Goodpasture, the first attempts were made with a strain of virus long cultivated *in vitro* in a medium consisting of monkey serum-Tyrode solution and minced chick embryo. After the culture flasks were incubated for 3 to 4 days at 37°C., the supernatant fluid was pipetted off and a drop deposited on the chorio-allantoic membrane of several eggs after a window-flap had been cut in the shell. At the end of a 4-day incubation period at 37°C., virus was demonstrable in considerable quantities by the intracerebral test in mice in both the embryonic fluid and in the embryo itself. It was found possible to passage this virus from egg to egg using the fluid, the membranes, or a suspension of embryonic tissue as the inoculum.

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[1] Woodruff, A. M., and Goodpasture, E. W., *Am. J. Path.*, 1931, **7**, 209.

[2] Haagen, E., and Theiler, M., *Zentralbl. Bakter., Abt. I, Orig.*, 1932, **125**, 145.

[3] Lloyd, W., Theiler, M., and Ricci, N. I., *Trans. Roy. Soc. Trop. Med. and Hyg.*, 1936, **29**, 481.

In order to simplify the technique of egg passage, a method of direct inoculation of the embryo itself was tried and was found to be satisfactory. Eggs having living embryos, preferably 7 days old, were selected. After cleansing the shell with 95% alcohol, a small hole was drilled with a sharp dissecting needle directly over the embryo as seen by the transmitted light. Care was taken not to pierce deeper than was necessary to puncture the outer membrane. Using a tuberculin syringe with a 1-inch 27-gauge needle, the embryo was stabbed by a quick thrust of the needle and 0.03 cc. of the virus-containing material was injected. After the needle was withdrawn, the small hole in the shell was sealed with liquid paraffin. The eggs were then incubated at 37°C. for the desired length of time and were turned every day in the customary manner.

After 4 or 5 days' incubation, the eggs were again examined by a transmitted light. Those containing living embryos were selected as a source of virus for further passages. After washing with 95% alcohol, the egg was placed in an egg cup with the air sac up. The shell was cut away with sterile scissors, the outer membrane ruptured, and the embryo lifted out by the neck. Usually 2 or 3 embryos were pooled for each passage. They were weighed, placed in a sterile mortar, chopped up with scissors, and then ground up thoroughly with sand. A 50% suspension was made of this triturated material in normal saline solution. The suspension was centrifuged for about 10 minutes at low speed to throw down the larger tissue fragments and the supernatant fluid used to inoculate a new series of eggs. A test for the presence of virus was made at each passage by inoculating a group of 6 mice intracerebrally with the supernatant fluid. At frequent intervals passage material was frozen and desiccated for preservation.

Strain of virus adapted to chick embryo tissues. The strain of yellow fever virus which was successfully maintained in developing chick embryo was one which had already been greatly modified in both its neurotropic and viscerotropic properties by prolonged cultivation *in vitro* in a medium consisting of chick embryo tissues in serum-Tyrode solution. Sixty consecutive egg-to-egg passages have been made with this virus. Successful transfers were made at intervals as short as 2 and as long as 8 days after inoculation. The optimum age of embryos for this purpose was found to be 7 or 8 days. When younger embryos were used, many deaths in the first 2 days after inoculation were encountered.

There is definite evidence to indicate that multiplication of the virus takes place in the embryo. The titer of the strain in the tissue cultures used to initiate this series was low, varying at different subcultures from 1 in 250 to 1 in 2000. The titer of the virus in the embryo tissue, on the other hand, was found to be 1 in 17,000 after 6 egg-to-egg passages, and 1 in 195,000 after 12 passages.

Strain of virus adapted to mouse embryo tissues. Another strain of yellow fever virus used in these experiments was one which had been cultivated continuously for a period of nearly 3 years in a medium consisting of mouse embryo tissue and 10% normal monkey serum in Tyrode solution. Developing chick embryos were inoculated with this strain in the manner described above and a multiplication of the virus in the embryos was readily demonstrated. Repeated titrations indicated that the virus concentration of this strain in tissue cultures varied from 1 in 5000 to 1 in 20,000. Infected chick embryos, on the other hand, proved to be infectious after 4 days of incubation in dilutions of 1 in 48,000 to 1 in 100,000, indicating a much higher concentration of virus than was observed in the tissue cultures.

Unmodified strains of yellow fever virus. Using the technique described above, 4 additional strains of yellow fever virus were successfully cultivated in developing chick embryo. Two of these strains, the Asibi and the French, were originally isolated in Africa and are highly pathogenic for laboratory animals. The other two, E-5 and AFB, were obtained from Brazil and possess only a mild degree of virulence. These unmodified strains were not so readily adapted to the developing chick embryo as were the tissue culture ones. Only one of them, the E-5, was successfully established at the first attempt, and subsequent transfers from egg to egg were continued without difficulty. With the remaining 3 strains, several attempts failed before they were successfully established and subsequent transfers to fresh embryos gave positive results. After a strain had been successfully carried through the first 2 or 3 passages, no difficulty usually was encountered in continuing its propagation in this manner. Transfers were made at 4- to 5-day intervals, at which time the virus had reached its highest concentration, although it was demonstrated in considerable quantities in embryos as late as 11 days after inoculation.

These strains of yellow fever virus have not been carried long enough in developing chick embryo to indicate whether a fundamental change in virulence and tissue affinity of the virus will eventually take place. In one test a Rhesus monkey was inoculated with the French strain of virus that had been carried through 10 egg-to-egg passages. This animal died promptly of typical yellow fever, indicating that no modification in the virulence of the virus had taken place as a result of serial passage through 10 developing chick embryos. In another test, a strain of virus, which had been carried through 165 subcultures in chick embryo tissue—normal monkey serum—Tyrode solution medium, and in addition passed through 18 serial direct transfers from one developing chick embryo to another, was tested in 4 normal Rhesus monkeys. These animals were inoculated intracerebrally with 1 cc. amounts of 1 in 5, 1 in 50, 1 in 500, and 1 in 5000 dilutions. Three of the 4 monkeys showed fever after an incubation period of 10 days. The one which had no fever showed signs of encephalitis on the 7th, 8th, and 9th days. Four weeks after the inoculation, their sera were tested for protective properties against yellow fever virus, and the animals were also given a test dose of highly virulent Asibi virus to determine their immunity. All 4 monkeys showed immune substances in their serum and all survived the test dose, indicating that they had become immunized as a result of the inoculation with chick embryo passage virus.

Conclusions. 1. Yellow fever virus, unmodified strains as well as those adapted to tissue cultures *in vitro,* can be successfully cultivated in developing chick embryo. 2. Direct transfers can be maintained by the use of the chorio-allantoic membrane, embryonic fluid, or a suspension of minced tissues of the embryo. 3. A method of direct inoculation of the embryo in the egg with a tuberculin syringe and fine needle has been found satisfactory for routine use. 4. The virus strains studied showed no evidence of modification in the relatively few passages so far made.

9159

Stability of Group-Specific Characteristics in a Hemolytic Streptococcus.

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The recent work of Lancefield[1] and others in establishing the host-origin of individual strains of hemolytic streptococcus is a distinct forward step in our knowledge of this group of organisms which is responsible for so many human and animal diseases. Lancefield's group A, determined by a precipitin-test, seems surely indicative of human origin, whether from carriers or from actual infections. Another property indicating a human strain is its specific power to dissolve human fibrin, as shown by the method of Tillett and Garner.[2]

Some uncertainty as to the stability of these characteristics has been raised by the experiments of Reich,[3] who apparently succeeded in transforming, by rabbit-passage, a human strain of streptococcus so that it lost its "human" specificity. On subculturing such a transformed strain, however, it reacquired the characteristics that we associate with human origin.

In view of these results, which differ in the technic involved from our own experiments, it seems worth while to report that a human strain ("H") of *Streptococcus pyogenes* (Holman), obtained 19 years ago from a case of human empyema following measles and bronchopneumonia, has retained all of its original characteristics as regards human source in spite of the fact that it has been passed, at irregular intervals, through the pleural cavity of no less than 187 rabbits. It has acquired a high pathogenicity for rabbits that it did not at first manifest, although this pathogenicity fluctuates somewhat with the age outside the body of the particular pleural fluid used for reinoculation. This strain "H" has been used for years in studying the mechanism of natural resistance and immunity to the streptococcus (Gay[4]). In short, this rabbit-passage strain of human origin not only conforms in biochemical characteristics to its original culture, but it retains its human characteristic of lysing human fibrin but not rabbit fibrin, and it precipitates with antiserum to Lancefield's group A. In other words, this passage-strain differs, since its isolation, only in the acquisition of a specific pathogenicity for rabbits, but it is still clearly identifiable as of human origin.

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[1] Lancefield, R. C., *J. Exp. Med.*, 1933, **57**, 571.

[2] Tillett, W. S., and Garner, R. L., *J. Exp. Med.*, 1933, **58**, 485.

[3] Reich, T., Proc. Soc. Exp. Biol. and Med., 1935, **32**, 639.

[4] Gay, F. P., *J. Am. Med. Assn.*, 1931, **97**, 1193.

9160

Blood Heparin and Lipid Amino N in Experimental Obstructive Jaundice.

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The hemorrhagic diathasis which accompanies obstructive jaundice has been a serious obstacle in the treatment of this condition. Petren[1] states that 2% of the cases of obstructive jaundice submitted to operation die of hemorrhage. In an analysis of the cases of obstructive jaundice in the New York Hospital over a period of 3 years, 50% of the operative deaths were due to hemorrhage. Walters[2] reported a similar experience.

Chemical analysis of the blood has failed to give any clue as to which patients would bleed following operation. Although the clotting time and bleeding time is increased in most instances there is no correlation between prolonged bleeding and clotting time and the hemorrhagic diathasis. Kirk and King,[3] Snell and Green,[4] Snell, Green and Rowntree,[5] Linton,[6] and Ravdin and Regal[7] concluded that the changes in the blood calcium are insufficient to explain the condition. Similarly, Moss,[8] Barke and Weir[9] found no significant variation in the fibrinogen. Doyen[10] states that the concentration of bile in the blood of subjects suffering from obstructive jaundice is insufficient to interfere with the clotting of blood. Bancroft[11] reported that the platelets of patients suffering from obstructive jaundice are normal in number and fragility. In an excellent review of the literature, Wangensteen[12] concludes that prediction of the hemorrhagic diathasis is not possible with the present methods.

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[1] Petren, G. V., *Bruns. Beiträge*, 1918, **110**, 237.

[2] Walters, W., *Surg., Gyn. and Obst.*, 1921, **33**, 651.

[3] Kirk, P., and King, C. G., *J. Lab. and Clin. Med.*, 1925, **2**, 928.

[4] Snell, A. M., and Greene, C. H., *Am. J. Physiol.*, 1930, **92**, 630.

[5] Snell, A. M., Greene, C. H., and Rowntree, L. G., *Arch. Int. Med.*, 1925, **36**, 273.

[6] Linton, R. R., *Ann. Surg.*, 1931, **93**, 707.

[7] Ravdin, I. S., and Regal, C., *Ann. Surg.*, 1930, **91**, 801.

[8] Moss, W., PROC. SOC. EXP. BIOL. AND MED., 1931, **29**, 328.

[9] Barke, C. F., and Weir, J. F., *J. Lab. and Clin. Med.*, 1933, **18**, 657.

[10] Doyen, M., *Compt. rend. de la Soc. de Biol.*, 1909, **66**, 442.

[11] Bancroft, F., Kugelmass, I., and Stanley-Brown, M., *Ann. Surg.*, 1929, **90**, 161.

[12] Wangensteen, O. H., *Ann. Surg.*, 1928, **88**, 845.

It is significant that two basic factors concerned with the clotting of blood have never been investigated. It is the purpose of this investigation to examine the heparin and the lipid amino N in the blood of experimental animals suffering from obstructive jaundice. The greater part of the lipid amino N is cephalin.

Heparin was extracted and assayed according to the method of Howell.[13] The potency of an extract of 100 cc. of blood as compared to similar amounts of physiological saline solution was tested by mixing these with normal blood and measuring the difference in clotting times. Twelve dogs were used in pairs. In most cases several control determinations were done on both members of a pair and then one member was subjected to ligation and division of the common duct under nembutal anesthesia. Beginning one week after operation, determinations were run weekly until the death of the jaundiced animal. Van den Bergh estimations and clotting times were also done. A statistical summary of the results is shown in Table I. The difference between the amount of heparin in the jaundiced animals and the controls is less than the standard deviation of each and therefore it may be concluded that there is no significant variation in the heparin in dogs suffering from obstructive jaundice.

TABLE I.
Blood heparin in the dog.

Type	No. animals	No. determinations	Mean*	Standard deviation
Control	10	33	6.95	9.17
Experimental	6	23	6.72	8.22

* This figure is the delay in minutes in the clotting of blood as described in the text.

Howell[14] states that the thromboplastic substance is cephalin. In these experiments lipid amino N of whole blood has been measured by the method of Kirk, Page and Van Slyke.[15] In 4 dogs, control determinations were run. These were then subjected to ligation and division of the common duct as before. Frequent determinations were made thereafter. All determinations were run on duplicate samples, no effort being made to have the samples of more than approximately similar weight. The results on one dog, as shown in Fig. 1, demonstrate a primary rise in the lipid amino N. This is followed by a progressive fall to a level far below the normal. This

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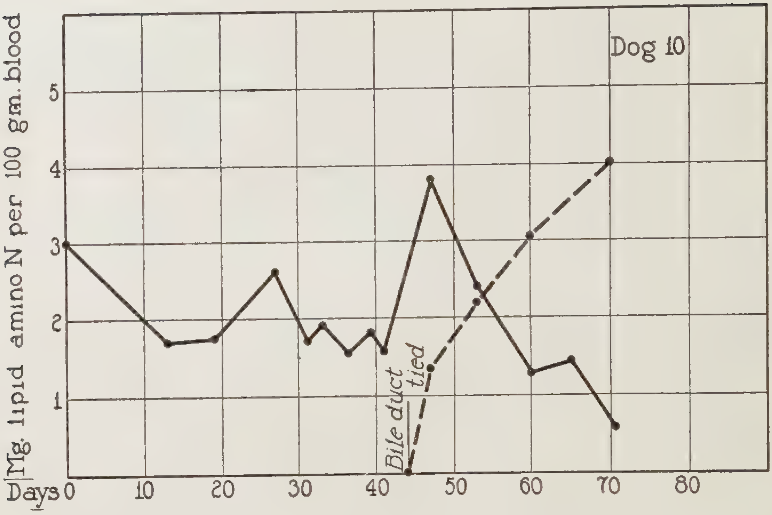
[13] Howell, W. H., *Am. J. Physiol.*, 1912, **31**, 1.

[14] Howell, W. H., *Am. J. Physiol.*, 1926, **77**, 680.

[15] Kirk, E., Page, I. H., and Van Slyke, D. D., *J. Biol. Chem.*, 1934, **106**, 203.



Lipid amino N in experimental ligation of bile duct

FIG. 1.

dog died 21 days after operation. In the second dog an attempt was made to do a cholecystoduodenostomy 33 days after the primary operation, at a time when the lipid amino N was low, and death occurred due to severe hemorrhage during the operation. In a third dog, there was a terminal rise in the lipid amino N and at autopsy there was a choledochoduodenal fistula and multiple abscesses of the liver. The fourth dog was similar to the first and second except that no initial rise after ligation of the bile ducts was seen.

In conclusion it has been shown that the circulating heparin is unchanged in obstructive jaundice in dogs. On the other hand, in uncomplicated cases there is a progressive fall of the lipid amino N of the blood, most of which is cephalin, the thromboplastic substance.

9161

Stimulation of Local Antibody Formation in Cornea by Grenz Rays.

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In a previous communication[1] we presented evidence that, when a solution of egg-albumin is injected into the corneal stroma, specific precipitins are produced locally. By pooling the tissues from several rabbits and the use of a capillary-tube technic, it was possible to measure the precipitin-concentration in the small amounts of juice obtained by subjecting the tissues to high pressure in a hydraulic press. Following the intracorneal injection of antigen, precipitins could be detected in the corneal tissues earlier and in greater concentrations than in the serum, in the other tissues of the eye, or in the spleen or bone marrow. When the albumin was injected into only one cornea of each animal no precipitins could be obtained from the uninjected corneæ. The removal of the corneal epithelium and endothelium before the tissue-juices were expressed did not affect the precipitin-titer.

Since a number of different rays of the electromagnetic spectrum are known to exert an influence on the healing of tissues, and Grenz rays, in particular, have been used for a number of years in this institution[2] and in several European eye clinics for the treatment of various corneal conditions it seemed desirable to determine whether antibody-formation in the cornea could be influenced by these rays. Grenz rays* are electromagnetic waves of about 2 Ångström units occupying a position in the electromagnetic spectrum between ultraviolet (1300 Å) and the Rœntgen ray (1/100-1/10,000Å). The waves of this length are not very penetrating and are absorbed by the superficial tissues. Bucky[3] has calculated that half of the radiation is absorbed in 0.47 mm. of water; in 0.52 mm. of muscle or *cutis vera;* in 0.56 mm. of epidermis and in 0.77 mm. of subcutaneous tissue. Approximately 60% of a 10 K.V.P. Grenz beam is able to penetrate 0.5 mm. of cutaneous tissue.

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[1] Thompson, R., Gallardo, E., Jr., and Khorazo, D., *Am. J. Ophth.*, 1936, **19**, 852.

[2] Pfeiffer, R., unpublished work, in Institute of Ophthalmology.

* Grenz rays are sometimes called Bucky's rays; Bucky designed the machine for their generation and has done much to establish their value as a therapeutic agent. See Bucky.[3]

[3] Bucky, G., Grenz Ray Therapy. 1926, Macmillan Co., N. Y.

The methods used in the preparation of the purified egg-albumin solution; for the corneal injections; the expression of the tissue-juices, and the technic for the titration of the precipitins by the optimal-proportions method were as described in the previous paper.[1] Before the Grenz ray treatments the rabbits' eyes were locally anesthetized by a few drops of 0.5% pantocaine. The lids, and when necessary the nictitating membrane, were retracted and the whole cornea exposed, so far as possible, to the rays. Each treatment† lasted for one minute. Four hundred and eighty-five R. were delivered with the following factors: 10 Ma., 11 K.V.P. and 9 cm. distance. The number and spacing of treatments are indicated in each experiment.

Experiment 1. Eight rabbits were injected into both corneæ on 2 successive days with 0.05 cc. of egg-albumin solution (containing approximately 9 mg. of N per cc.). The corneæ of the right eyes were exposed to the Grenz rays for one minute on the day of the second injection and on the succeeding 3d, 6th, and 11th days. The left eyes of all animals were subjected to the same pantocaine drops and to the one-minute exposure to drying as the right eyes, the rays being omitted. Fifteen days after the first injection of antigen the animals were killed by bleeding and the tissue-juices and serums titrated for precipitin content.

Experiment 2 was similar to Experiment 1 except that 5 ray treatments were given and a 14-day interval elapsed between injections and killing.

Experiment 3. A group of 8 rabbits were given Grenz ray treatments of the right corneæ on alternate days for 8 days. On the 10th day 0.05 cc. of egg-albumin was injected into 2 places in both corneæ of all rabbits. A fifth treatment of the right corneæ was given the following day. The rabbits were killed 14 days after the injections.

The titrations of precipitin in these 3 experiments are shown in Table I.

The rayed corneæ consistently contained greater concentrations of precipitins than did the untreated corneæ. The difference was most marked in Experiment 3 in which the Grenz ray treatments were begun 8 days before the injection of antigen; the titer of the treated corneæ being approximately 12 times that of the untreated. The increased precipitin concentration in the corneæ treated by rays of such little penetrating power is additional evidence for the local

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† We are indebted to Mrs. D. Albert, X-ray technician of the Institute, for her coöperation.

TABLE I.

Comparative precipitin-titers of treated and untreated corneæ. (Optimal-proportions method.)

Experiment		Quantity* of Precipitate in Tubes Containing Various Concentrations of Egg-albumin (mg. of nitrogen per cc.)											
		.08	.07	.06	.05	.04	.03	.02	.01	.005	.0025	.001	Saline
1	Treated Corneæ	0	0	0	1	1	4	6	8	8	4	2	0
	Untreated Corneæ	0	0	0	0	0	0	0	2	4	4	2	0
	Pooled Serums†	0	0	0	0	0	0	0	0	0	0	0	0
2	Treated Corneæ	4	4	8	8	8	12	16	16	12	8	4	0
	Untreated Corneæ	trace	2	2	2	2	4	8	8	8	8	1	0
	Pooled Serums†	0	0	0	0	0	0	0	0	0	0	0	0
3	Treated Corneæ	7	8	12	10	10	9	8	8	8	2	2	0
	Untreated Corneæ	trace	trace	1	1	1	1	2	2	4	2	trace	0
	Pooled Serums	0	0	0	0	0	0	0	0	0	0	0	0

* Each 0.25 mm. of column of precipitate considered as 1 unit.

† When the serums were tested individually 2 serums in both experiments 1 and 2 showed minute traces of precipitins.

Experiment 3 was done a year later than the others. The tube had not been calibrated before this series, so that the R units may have varied somewhat.

production of the precipitins since the corneal tissue must necessarily be the only tissue affected. In an attempt to determine whether the ray treatment accelerated precipitin formation 2 experiments were done in which the intervals between injection of antigen and killing were respectively 7 and 10 days, in contrast to the 14-day intervals of the previous experiments. Five Grenz ray treatments were given following the injections of antigen. In both experiments antigen was still present, both in the treated and untreated corneæ, and no precipitins were shown. We have not yet determined whether treatment before injection of antigen, shown in Experiment 3 to be most effective, would produce an accelerated formation of antibodies.

We have not found any previous work dealing with the effect of Grenz rays on antibody-formation or of any type of electromagnetic wave upon *local* antibody-formation. General exposure to ultraviolet rays has been reported as stimulating the production of agglutinins,[4, 5] and of hemolysins.[6] The effect of general exposure to

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[4] Hansen, T., *Compt. Rend. Soc. Biol.*, 1921, **85**, 1199.

[5] Bassemans, A., and Seldeslachts, A., *Compt. Rend. Soc. Biol.*, 1929, **100**, 1055.

[6] Omar, W., *J. Egyptian Med. Assn.*, 1935, **18**, 168.

Rœntgen rays has repeatedly been described as a diminution of antibody-production due to injury of the bone marrow, spleen or lymphatic tissues.[7-10]

Summary. The local formation of precipitins following the injection of purified egg-albumin into the rabbit's cornea was increased by exposure of the cornea to the dosages of Grenz ray used in these experiments. The effect was most marked when the exposures were made for several days prior to injection of antigen.

9162

Factors Influencing Sedimentation Rate of Erythrocytes.

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There is increasing use of the sedimentation rate of erythrocytes as a test in clinical medicine, but there is equal difficulty in correct interpretation of the results obtained due to the many factors that influence it. Much work has been done to elucidate the value of this test in different clinical conditions, especially in infections.

Fahareus[1] has reviewed the literature and studied many of the factors that influence the sedimentation velocity of erythrocytes, especially the amount of fibrinogen and globulin in the plasma. Many other publications add new evidence. Westergren[2, 3] states that a definite positive correlation exists between the plasma proteins and the sedimentation of erythrocytes. Zarday and Farkas[4] have modified blood by the addition of normal fibrinogen and globulin and found that the sedimentation rate was increased in proportion to the amounts added. When they added albumin the rate was decreased. Coburn and Kapp[5] arrived at similar conclusions and added that

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[7] Benjamin, E., v. Sluka, E., *Wien. Klin. Wchnschrift*, 1908, **21**, 311.

[8] Läwen, 1909, quoted by Hektoen.[9]

[9] Hektoen, L., *J. Inf. Dis.*, 1915, **17**, 415.

[10] Murphy, J. B., and Sturm, E., *J. Exp. Med.*, 1925, **41**, 245.

[1] Fahareus, R., *Physiol. Rev.*, 1929, **9**, 255.

[2] Westergren, A., Theorell, H., and Widström, G., *Z. f. d. g. exp. Med.*, 1931, **75**, 668.

[3] Westergren, A., Juhlin-Dannfelt, C., and Schnell, R., *Acta Med. Scand.*, 1932, **77**, 469.

[4] Zarday, I., and Farkas, G., *Z. f. d. g. exp. Med.*, 1931, **78**, 367.

[5] Coburn, A. F., and Kapp, E. M., *J. Clin. Invest.*, 1936, **15**, 715.

albumin inhibited sedimentation almost completely. Lucia and coworkers[6–9] found that *in vitro* a consistent relationship cannot be established between the sedimentation rate and the globulin content of various dilutions of serum globulin. They also showed that *in vivo* the sedimentation rate is apparently accelerated as the globulin content of the serum increases, and that a cause and effect relationship between the quantity of the various serum proteins and the rate of sedimentation cannot be said to exist. Further, they have shown that beef serum albumin or human serum albumin prolonged the sedimentation time when dissolved in Locke's solution or added to plasma.

Such controversial statements are found in the literature concerning the effects of the different protein fractions on sedimentation rate.

We have briefly attempted to show some of the fundamental factors that influence sedimentation rate of erythrocytes. The method used in determining the sedimentation velocity is as follows: Whole oxalated blood was used. The Weintrobe hematocrit tube was filled to 1 cc. mark, the rate of sedimentation observed, the reading being recorded every 15 minutes up to 2 hours, when the final reading was taken. This last figure is here reported in millimeters.

Effect of Number of Erythrocytes. We made 2 sets of experiments, varying the number of erythrocytes, using the same plasma, with the following results:

A		B	
No. of cells	Sed. rate	No. of cells	Sed. rate
5,600,000	5.0	7,000,000	1.0
4,200,000	24.0	4,200,000	29.0
2,800,000	48.0	3,200,000	49.0
1,400,000	79.0	2,100,000	68.0
		1,050,000	85.0

These results show that there is a logarithmic relationship between the number of erythrocytes and the sedimentation rate, a result that might be expected from theory. It becomes of importance to report the blood count in showing the sedimentation rate, so that the results of one test can be compared with another.

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[6] Lucia, S. P., and Brown, J. W., PROC. SOC. EXP. BIOL. AND MED., 1934, **32**, 189.

[7] Lucia, S. P., Gospe, S. M., and Brown, J. W., PROC. SOC. EXP. BIOL. AND MED., 1935, **33**, 356.

[8] Lucia, S. P., Blumberg, T., Brown, J. W., and Gospe, S. M., *Am. J. Med. Sci.*, 1936, **192**, 179.

[9] Lucia, S. P., and Brown, J. W., PROC. SOC. EXP. BIOL. AND MED., 1936, **35**, 598.

Effect of the Different Plasma Protein Fraction. Two sets of experiments were done, with the different protein fraction in similar concentrations, using approximately the same number of erythrocytes in each case, but from 2 different individuals. The results are as follows:

	A	B
Protein fraction	Sedimentation rate	
Fibrinogen	41.0	61.0
Euglobulin	42.0	64.0
Pseudoglobulin	5.0	12.0
Albumin	3.0	6.0

These results agree in general with those reported by different authors, except that in our case the euglobulin had a more accelerating effect than the fibrinogen, but for general consideration we can assume that their effect was similar. The graduation of the accelerating effect of the different protein fractions suggests strongly that there is a parallel effect to the electric charge of these proteins, it being that the less charge, the accelerating effect becomes more marked. The cells are more closely packed and the rouleaux are more tight, and often there is definite aggregation of the cells in strong globulin solutions. In the albumin solution, on the contrary, the cells are seen dispersed, the rouleaux are either very loose or broken, the shape of the cells often showing distortions when seen under the ultramicroscope.

Effect of Albumin-Globulin Ratio and Total Protein. Wishing to test the statement of several authors as to the positive correlation between the plasma proteins and sedimentation velocity, we determined the index of correlation with 100 cases between the albumin-globulin ratio and the sedimentation rate. We also correlated the total protein percent with the sedimentation rate. Our results are shown in Table I. There is a negative correlation without significance between the albumin-globulin ratio and the sedimentation rate, the same being true with the total protein percentage. The large value for sigma (standard deviation) in the sedimentation rate and the albumin-globulin ratio in proportion to the mean average further demonstrate the broad distribution of the observed data.

TABLE I.
Index of correlation between sedimentation rate and A/G ratio.

	Mean Aver.	σ	Index of Corr.
Sed. Rate (x)	14.3	13.60	0.04(x-y)
A/G (y)	2.0	1.28	
T.P. (z)	7.20	0.67	0.08(x-z)

A/G Albumin-globulin ratio.
T.P. Total protein percentage.

Effect of Different Metal Ions. To avoid the Ca effect on coagulation we used heparin as an anticoagulant in these experiments. To 1 cc. of the blood we added 0.1 cc. of a 0.1 M solution of the chloride salt of Na, K, Ca, and Mg. The results here given are the average of many experiments:

Na—7.0; K—5.8; Ca—5.4; Mg—3.3

The inhibiting effect of the different ions follows the Hofmeister series, increasing with the series. The effect of valency seems to explain only partly the results observed, for it seems that there is a specific effect by the metal ion itself.

Effect of Lecithin and Cholesterol. Suspensions of purified lecithin and cholesterol were made, and equal amounts (0.8 mg.) were added to the various tubes, keeping a control to which the same amount of Locke's solution was added. Table II shows the results of these experiments.

TABLE II.
Effect of lecithin and cholesterol on sedimentation rate.

Control	22.5	21.5	21.0	25.0	10.0	22.4	17.0	31.0	16.0	4.0
Lecithin	14.0	1.5	2.0	1.2	1.5	8.9	14.8	18.6	8.0	2.1
Cholesterol	24.8	29.0	21.5	21.8	11.0	23.5	18.5	32.2	17.0	4.4

These results show the marked retardation produced by lecithin and to a less extent the accelerating effect of cholesterol. Under the ultramicroscope we observe the dispersion of the red cells and the modification of their shape (spherical) produced by the lecithin, while the cholesterol increased the closeness of the rouleaux. This difference in effect is probably due to the change in electrical charge and hydration of the red cells by these substances. The intensity of effect on the different bloods produced by the same amount of the lecithin and cholesterol is difficult to explain.

Effect of Erythrocytes from Different Individuals in the Same Plasma. The blood was centrifuged and the corpuscles washed with Locke's solution, then equal amounts of erythrocytes were measured in each one of the different tubes that contained an equal amount of plasma. The results of the sedimentation rate are shown in Table III.

The results show that there is a notable difference in the different erythrocytes. Whether this difference is due to chemical nature

TABLE III.
Effect of different erythrocytes on the same plasma.

Case	1	2	3	4	5	6	7	8	9	10
Sed.	8.6	24.6	7.2	15.0	32.0	26.6	19.0	1.8	8.0	6.5

or not is difficult to say, but it is more probable that there are differences in the size, charge and hydration of the corpuscles. In order to study this problem further, we studied the effect on sedimentation rate of the same erythrocytes in different plasmas. We give the refractive index and the viscosity of each one of the plasmas to give a general idea as to their protein composition. Table IV gives the result of this experiment.

TABLE IV.
Effect of the same erythrocytes on different plasmas.

Case	1	2	3	4	5	6	7	8	9	10
R. I.	1.347	1.350	1.350	1.347	1.348	1.348	1.346	1.348	1.346	1.350
Vis.	1.73	2.02	2.02	1.72	1.77	1.66	1.77	1.77	1.72	2.08
Sed.	8.6	43.8	29.6	13.0	21.0	12.0	33.0	25.4	49.6	26.8

Here we observe a similar difference from the one observed above. There is a marked difference between the effect of the various plasmas on the same erythrocytes, suggesting that there are other factors that influence the sedimentation rate than the ones already considered.

Effect on Sedimentation from Erythrocytes of Different Species on its Homologous and Different Plasmas. The plasma was separated from the blood of a horse, an ox, and a human case, and the cells washed with Locke's fluid several times. Equal amounts of plasma from the different specimens were placed in tubes to which equal amounts of cells suspensions from each individual were added. A count was made to determine the number of erythrocytes in the final dilution and the mixtures were placed in the hematocrit tubes. The result of this experiment is shown in Table V.

TABLE V.
Effects of different erythrocytes on different plasmas.

Erythrocytes Number	Human 3,100,000	Horse 4,530,000	Ox 3,200,000
Human Plasma	40.0	41.2	2.0
Horse ”	56.5	52.5	3.0
Ox ”	60.2	36.5	1.4

This experiment clearly demonstrates that ox corpuscles are much slower in sedimenting than horse or human cells in the various plasmas. Human erythrocytes sediment faster in the different plasmas than either horse or ox, but more marked in ox plasma. The reason for the horse cells sedimenting slower in ox plasma is not clear. It is clearly demonstrated that there are intrinsic factors in the plasma and on the erythrocyte that affect sedimentation velocity which can not be predicted, making the sedimentation test theoretically difficult to evaluate.

9163 P

Maintenance of Life in Adrenalectomized Animals with Flavin Phosphate.

M. Pijoan and S. A. Oberg. (Introduced by E. C. Cutler.)

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The recent work of Verzár and Laszt[1] has suggested that the possible function of cortin in the animal body is to convert protovitamin B_2 (Flavin) to Vitamin B_2 (Flavin phosphate). The mortality rate of their adrenalectomized animals was materially reduced by injections of Flavin phosphate. It is the purpose of this communication to confirm the work of Verzár and Laszt[1] and to present additional data.

Normal rats were adrenalectomized and divided into several series of 15 rats each as follows: (1) Rats receiving 0.15 cc. Flavin phosphate daily, (2) rats receiving 0.03 cc. Flavin phosphate daily, (3) rats receiving 0.03 cc. Flavin phosphate and 50 mg. ascorbic acid daily, (4) those receiving 0.25 cc. cortin and those (5) receiving no medication. There were also control series. The Flavin phosphate was prepared by the method of Karrer, Salomon, Schopp and Benz[2] and Kuhn and Rudy[3] and also by applying Kuhn's method to yeast which Stern[4] used to extract photoflavins from liver. The rats were placed on the Flavin free diet of Verzár and Laszt[1] whereas another group was placed on the ordinary preoperative rat diet. The results can be summarized as follows: Rats receiving 0.15 cc. daily of Flavin phosphate maintained normal weight and activity for 3 weeks and were finally autopsied to confirm adrenalectomy. Seventy-five per cent of the rats receiving the smaller dose of 0.03 cc. of Flavin phosphate died within 10 days, the remainder dying within 3 weeks. The rats receiving 0.03 cc. of Flavin phosphate and 50 mg. ascorbic acid (Hoffman-LaRoche) survived 3 weeks, losing weight gradually and died at the end of 4 weeks. Those receiving 0.25 cc. cortin died on the Flavin free diet, but maintained fairly good health on the ordinary diet. Those receiving cortin and ascorbic acid behaved as those receiving cortin alone. Those receiving no medication died within 4 days.

The data suggest that there is a synergistic action between ascorbic acid and Flavin phosphate (Vitamin B_2) and that cortin cannot maintain the life of adrenalectomized rats on a Flavin-free diet. Adrenalectomized rats can be maintained in good condition with Flavin phosphate. Young adrenalectomized rats furthermore have normal growth curves if given Flavin phosphate. Studies are being carried out in cats and dogs after adrenalectomy in the relation of Flavin phosphate to maintenance of life and general metabolism.

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[1] Verzár, F., und Laszt, L., *Pflüger's Arch. Physiol.*, 1936, 476.

[2] Karrer, P., Salomon, H., Schopp, K., and Benz, F., *Naturwiss*, 1935, **23**, 355.

[3] Kuhn, R., and Rudy, H., *Ber. Deutsch. Chem. Ges.*, 1935, **68**, 383.

[4] Stern, K. G., *J. Biochem.*, 1934, **28**, 949.

9164 P

Thyro-Parathyroidectomy and Pregnancy in the Rat.

M. BODANSKY AND W. R. COOKE. (With the assistance of Cornelius L. Herrmann and V. B. Duff.*)

From the Laboratories of the John Sealy Hospital and the Departments of Obstetrics and Gynecology and Pathological Chemistry, University of Texas School of Medicine, Galveston.

In studying the metabolism of thyro-parathyroid deficient rats it was found that a number of the animals which had become pregnant died at term, often during labor, death being preceded by convulsive seizures, occurring at intervals. This recalled the remarkable observation published by Halsted[1] in 1896, before the function of the parathyroids was known. Working with dogs, Halsted performed piecemeal extirpation of the thyroid and studied the changes produced in the remnants of the gland. He was particularly interested in determining the validity of Munk's contention that the thyroid was a superfluous organ. The dogs usually survived repeated operations and did not develop the fatal symptoms resulting from complete removal of the gland. However, 2 of the partly "thyroidectomized" dogs which had become pregnant developed tremors and convulsions at term, these symptoms subsiding after the pups were born.

Our observations seemed of sufficient importance to justify more detailed investigation and accordingly the study was extended to a group of 62 thyro-parathyroidectomized rats, this series being the basis of the present report. Total extirpation of the glands was performed at a relatively early age (less than 110 days, except in 6, operated at 135-140 days). The very young were allowed to ma-

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* Fellow, 1936-37, under a grant from Mead Johnson and Company in support of research in the Department of Pathological Chemistry.

[1] Halsted, W. S., *Johns Hopkins Hosp. Reports*, 1896, **1**, 373.

ture and to attain a weight of 160-180 gm. before mating; the others were mated a few days after the operation. All the rats were maintained in an apparently good state of nutrition. As the subsequent course of events did not seem to be influenced by the administration of maintenance doses of thyroxine (Hoffmann-LaRoche), or desiccated thyroid (Eli Lilly), it seems unnecessary to dwell on the details in this communication.

Of the 62 operated rats, 42 did not survive one or more pregnancies, although they appeared in good health until nearly the end of gestation. Death occurred at term, either before or after the onset of labor. In many, tetany preceded the later semi-moribund condition. In others, tetany may have been overlooked, as the rats were not regularly inspected during the night. The results are briefly summarized in Table I.

TABLE I.

		Ratio of matings to pregnancies	Ratio of rats successfully reared to number born
Total number of thyro-parathyroidectomized rats	62		
Rats succumbing at end of first pregnancy	24	98/69	63/199†
’’ ’’ ’’ ’’ ’’ second ’’	12		
’’ ’’ ’’ ’’ ’’ third ’’	3		
’’ ’’ ’’ ’’ ’’ fourth ’’	3		
Number of surviving rats	20‡		
Surviving rats remaining sterile after repeated matings	3		
Surviving rats pregnant once	5	113/38	109/284
’’ ’’ ’’ twice	7		
’’ ’’ ’’ three times	3		
’’ ’’ ’’ five ’’	2		
Normal controls	16	57/44	159/387
Rats repeatedly and successfully pregnant	15§		

† In addition 411 mature fetuses were counted *in utero* and 33 more were born before the death of the mothers. In 2 rats several days past term, post-mortem examination disclosed partial fetal resorption.

‡ Of these, one was under observation 5½ months, 8 for 7½ months, and the remaining 11 for 10 to 11 months.

§ One of the normal controls died at the end of its second pregnancy. This was unusual, having occurred only 2 other times in approximately 1000 pregnancies in our colony.

From the data outlined above it will be noted that the proportion of successful matings was lower in the surviving than in the non-surviving group. It is obvious that this in itself assured a lower risk of mortality in the less fertile rats. Thyro-parathyroid deficiency was established in all the animals by the following criteria: (1)

failure to find remnants of thyroid or parathyroid tissue at autopsy; (2) failure in the normal metabolic response on exposure to cold, as described elsewhere;[2] (3) negligible excretion of guanidoacetic acid;[2] (4) low serum calcium (4-6.5 mg.) in every case where blood was available for analysis.

Summary and Conclusions. In pregnant rats deprived of their thyroid and parathyroids a very high rate of mortality occurred at term. While it seems logical to attribute these results to parathyroid deficiency, and indeed more recent experiments in this laboratory support this conclusion, nevertheless other factors may be of importance. Work is in progress in an attempt to elucidate the problem.

9165

Effect of Progesterone on Cell-Division in the Uterine Epithelium.

CHARLES W. LLOYD. (Introduced by George W. Corner.)

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It has been stated (see, for example, Sex and Internal Secretions, edited by E. Allen, 1932, p. 528) that the corpus luteum hormone, progestin, does not cause active proliferation with mitotic division in the endometrium, the proliferation observed after ovulation being ascribed to the action of oestrin and not progestin.

Examination of a cross section of the rabbit's uterus after injection of the castrated animal with progestin for five days, as in the usual method of assay, reveals very little evidence of mitotic division; it is, no doubt, this observation which has given rise to the conclusion that progestin does not induce cell division. The great increase, however, in extent of the surface epithelium and gland cells which is seen in the sections, obviously depending upon an increase in the total number of cells, suggests that cell-division has in some way been induced.

In the course of the experiments reported by Makepeace, Corner and Allen[1] it was noted that the uteri of animals examined after 3 days of progestin treatment showed frequent mitoses in the epithelium. Probably, therefore, when the injections of progesterone are continued for 5 days, as in the experiments of other workers, the endometrium passes beyond the division stage, and by the time of autopsy all of the mitotic figures have disappeared.

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[2] Bodansky, M., *et al.*, *Endocrinology*, 1936, **20**, 822.

[1] Makepeace, A. W., Corner, G. W., and Allen, W. M., *Am. J. Physiol.*, 1936, **115**, 376.

The following experiment was done to test this conjecture under controlled conditions. Complete oöphorectomies were done on 6 rabbits. At the time of operation, a specimen was removed from the middle of one horn of each uterus as a control. Two of the rabbits each received daily for 3 days 0.5 mg. progesterone (crystalline progestin), an amount comparable with that produced in a rabbit with active corpora lutea. Two rabbits each received 3 daily doses of 50 international units of oestrin in the form of Amniotin in oil. Two animals were kept as controls and received no injections.

The animals were killed on the fourth day after operation. Specimens from the uteri were taken at autopsy, as nearly as possible from the middle part of the horn not used as the control. The sections were stained with iron hematoxylin and the mitotic figures were counted in two sections for each rabbit, one taken at operation and one taken at autopsy. The results obtained are shown in Table I.

TABLE I.

Treatment	Number of Mitoses At operation	 At autopsy
Control	6	12
,,	7	8
Oestrin	8	13
,,	12	15
Progesterone	9	ca. 500
,,	12	ca. 750

The difference between the number of mitotic divisions in the oestrin-injected animals and in the control animals is so slight as to be negligible. On the other hand, the ratio of the number of figures found in sections from the progesterone-injected animals to the number found in oestrin-injected animals is at least 50:1. The conclusion to be drawn from these results is that an initial effect of progesterone is to stimulate cell-division of the uterine epithelium.

Thanks are due to Dr. Willard M. Allen for provision of the progesterone.

9166

Failure of Histamine and "Gastrin" to Provoke Gastric Secretion in Monkeys with Anacidity.

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In the course of gastric analysis upon healthy Rhesus monkeys, it was found that 3 of 12 monkeys failed to secrete free hydrochloric acid following a meal, an alcoholic test meal, and also subcutaneous injections of histamine in dosages up to one milligram.

Since these animals were not responsive to histamine, it was thought that they might respond to "gastrin" or an extract of pyloric mucosa; and if so, we would have proof that "gastrin" and histamine are not identical[1] and also that the anacidity of the 3 monkeys is due to the absence of the "gastrin" mechanism.

Gastric analysis was performed upon 12 healthy Rhesus monkeys *(Macaca mulatta)* which had been fasted for 24 hours. A Rehfuss tube (minus the olive) was passed into the stomach and the fasting contents were removed; the stomach was washed with distilled water and emptied. The monkeys then each received 0.5 mg. histamine (ergamine acid phosphate) subcutaneously. If free acid failed to appear in the gastric samples, the dosage of histamine was increased to 1.0 mg. Gastric samples were taken 45 to 60 minutes after each injection and analyzed for free acid (p-dimethylaminoazobenzene) and bile (naked eye). "Gastrin" extracts of pyloric mucosa (pig) were prepared after the method of Keeton and Koch.[2] The quantity of "gastrin" equivalent to one mg. of histamine was determined by subcutaneous injection in a dog with a pouch of the whole stomach.[3] The vaso-depressor activity of the "gastrin" as compared to histamine was determined by intravenous injection in the dog.

Hemoglobin, hematocrit and red blood cell determinations were made on all of the monkeys.

The viscid, mucoid fasting contents of the stomach were scanty, never contained free acid and but little total acid.

None of the monkeys secreted free acid in response to 7% alcohol or to a meal of farina gruel and boiled potatoes.

Six out of 12 monkeys failed to develop free acid in their gastric juice 45 to 60 minutes after subcutaneous injections of 0.5 mg. histamine. Three out of 12 monkeys (Nos. 5, 10, 12) showed no free acid when the dosage of histamine was raised to 1.0 mg.

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[1] Sacks, Ivy, Burgess and Vandolah, *Am. J. Physiol.*, 1932, **101**, 331.

[2] Keeton and Koch, *Am. J. Physiol.*, 1915, **37**, 481.

[3] Lim, Ivy and McCarthy, *Quart. J. Exp. Physiol.*, 1925, **15**, 13.

Two cc. of the "gastrin" solution given subcutaneously resulted in a stimulation of gastric secretion in the dog with a pouch of the whole stomach quantitatively similar to 1.0 mg. histamine. One cc. of the "gastrin" solution given intravenously had a vaso-depressor action in the dog quantitatively similar to 0.5 mg. histamine.

Five out of 12 monkeys failed to show free gastric HCl following 1.0 cc. of "gastrin" solution. The same 3 monkeys (Nos. 5, 10, 12) that failed to respond to 1.0 cc. histamine also failed to respond to 2.0 cc. of the "gastrin" solution.

In the monkeys showing anacidity the gastric secretions were scanty (2 cc.), thick and viscid, resembling the fasting secretion.

The blood studies showed that none of the monkeys were anemic at the time these studies were made.

If "gastrin" and histamine are different and if "gastrin" rather than histamine is the gastric secretory hormone, one might expect a stimulation of gastric secretion in the monkeys refractory to histamine. A positive response to "gastrin" and a negative response to histamine would have been very significant. Since a positive response to "gastrin" was not obtained, the parietal cells of the 3 anacid monkeys are refractory to all ordinary stimuli. These results add an indirect point of evidence supporting the conclusion of Sacks, Ivy, *et al.*,[1] that histamine is the sole gastric secretory excitant in acid extracts of pyloric mucosa.

Summary and Conclusions. 1. Six out of 12 Rhesus monkeys failed to secrete free acid in their gastric juice 45 to 60 minutes after subcutaneous injections of 0.5 mg. histamine while 5 out of 12 failed to respond to 1.0 cc. of a preparation of "gastrin". 2. Three out of 12 monkeys were refractory to 1.0 mg. of histamine and to 2.0 cc. of the "gastrin" preparation. 3. The "gastrin extracts" of hog's pyloric mucosa did not contain a substance capable of stimulating acid gastric secretion other than histamine, both in the normal and anacid monkeys. 4. None of the 12 monkeys secreted free acid in the gastric juice in response to 7% alcohol or to a meal of farina gruel and boiled potatoes.

9167 P

Urinary Excretory Rate of Adrenalectomized Rats.

R. D. Beaird, Jr., and H. G. Swann. (Introduced by A. J. Carlson.)

From the Department of Physiology, University of Chicago.

The excretory rates of 10 normal mature rats were followed for 15 days on the following diet: 0.5% solution of sodium salts (consisting of 7 parts NaCl to 3 parts $NaHCO_3$) for drinking water; 25 cc. of milk (canned, Carnation) and one-half of a slice of white bread daily; "Fox Chow" kept in the cages at all times as a staple.

After 15 days, bilateral adrenalectomy was performed on 6 of these animals and sham operations on the other 4, these being kept as controls. The diet used has been shown to preserve the lives of adrenalectomized rats for a month or more.[1, 2, 3] The rats after operation were in good condition, gaining an average of 0.31 gm. per day during the 3 weeks after adrenalectomy.

Each rat's cage rested on supports inside the edge of a 12-inch milk funnel. The urine was collected in a beaker fastened to the base of the funnel. Food was placed in dishes set into the side of the cage as is done in bird cages.

All adrenalectomized rats except one showed on autopsy no macroscopic adrenal tissue. This animal survived in good health for 22 days after withdrawal of the bread and milk and the salt drinking water. When sacrificed, autopsy showed a piece of adrenal tissue 3 mm. in diameter. This rat's excretory rate did not vary from normal.

The remaining 5 adrenalectomized rats were observed for 23 days. Then the sodium solution and the bread and milk were withdrawn from the diet, leaving "Fox Chow", with occasional lettuce and meat scraps as the diet and tap water as the drinking solution. Three of these animals died at 6 days after the change in diet, one at 9 days and one at 10 days.

The urinary excretory rate of normal rats, as measured by our technique, was about 4 cc. per day. After adrenalectomy, if life were preserved by means of the bread and salt diet, the rats developed a considerable diuresis, each rat excreting about 20 cc. per day.

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[1] Cleghorn, R. A., Cleghorn, S. M. M., Forster, M. G., and McVicar, G. A., *J. Physiol.*, 1936, **86**, 229.

[2] Swann, H. G., *Am. J. Physiol., Proc.*, 1936, **113**, 129.

[3] Swann, H. G., *Am. J. Physiol.*, in press.

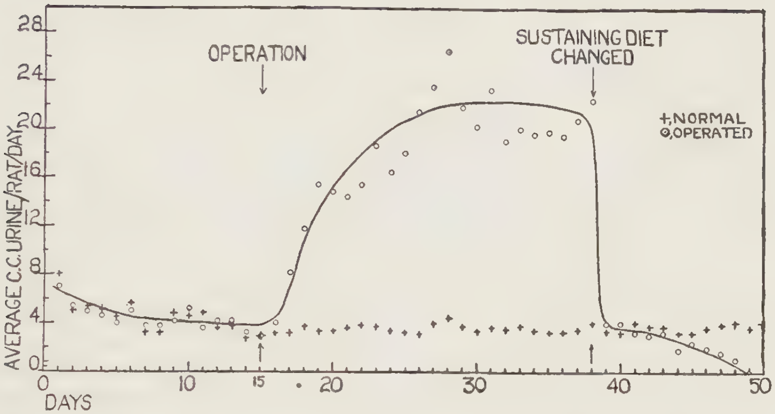

Fig. 1.
Urine excretion rates of normal and adrenalectomized rats on identical diets.

This is approximately 5 times the normal rate. The controls during this period did not vary from the normal excretory rate that had previously been established. When the life sustaining diet was removed from the operated animals, the polyuria disappeared within 24 hours. Subsequently, as death approached, the excretory rate dropped to subnormal. When the unoperated controls were subjected to the same dietary regime, including all the changes, their excretory rates did not vary appreciably.

The data of 5 adrenalectomized rats (Fig. 1) are here included. In other studies of 12 adrenalectomized rats maintained in good health by dietary measures, the excretory rates of rats have been measured. These rats all conform to those which are here reported.

That we are dealing here with a relatively large diuresis is indicated by the following facts: removal of the posterior hypophysis in rats causes a long-continued diuresis.[4, 5] When the excretory rates of such rats (24 in number) are measured by our technique, it is found that they excrete about 12 cc. per day. This is considerably less than the daily excretion here reported after adrenalectomy, *viz.*, 20 cc. In one extreme case of an adrenalectomized rat (not included in this group), the urinary output was about 48 cc. daily during one week.

Silvette and Britton have reported[6] that the excretory rate of rats in adrenal insufficiency is less than normal. This we have confirmed. Jiminez similarly reported[7] that the terminal stages of adre-

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[4] Richter, C. P., *Am. J. Physiol.*, 1934, **110**, 439.

[5] Pencharz, R. I., Proc. Soc. Exp. Biol. and Med., 1936, **34**, 14.

[6] Silvette, H., and Britton, S. W., *Am. J. Physiol.*, 1933, **104**, 399.

[7] Jiminez, D., *Abstr. J. Am. Med. Assn.*, 1936, **107**, 1422.

nal insufficiency in humans are accompanied by an oliguria. This report indicates that diuresis accompanies relief from adrenal cortical insufficiency.

Summary. After adrenalectomy, rats given a life-sustaining diet show a urinary excretory rate approximately 5 times normal. When the life-sustaining diet is changed to a non-sustaining diet, the excretory rates drop to normal and below, with death ensuing.

9168

Renal Blood Flow After Subtotal Nephrectomy.

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Arterial hypertension has been produced in animals by two methods of damaging the kidneys, (a) by renal ischemia due to a clamp which narrows the lumen of the renal artery,[1] and (b) by subtotal nephrectomy.[2, 3] With the latter method in the rat, observations have shown that in the first month after operation there is no hypertension although the amount of renal tissue is at a minimum (100 mg. per 100 sq. cm.) and there are polyuria and increased blood urea concentration (200 mg. %). Hypertension does not appear until the second month, when there are proteinuria and greatly hypertrophied renal stumps (200 mg. per 100 sq. cm.).

If the renal tissue had hypertrophied more rapidly than its vascular supply, thus causing a relative ischemia, the mechanism of the hypertension would be analogous to that in dogs with a clamp which narrows the lumen of the renal artery. To test this hypothesis, the rate of renal blood flow was studied.

Methods. Subtotally nephrectomized[2, 3] rats 2 weeks and 5 months post-operatively and unilaterally nephrectomized rats were used. Under ether anesthesia the abdomen was opened. Because of renal-splenic adhesions the gastro-splenic ligament was divided between ligatures; veins from the adrenal, leg, and testis were tied. The aorta and inferior vena cava were tied just below the entry of the left renal vein. Finally the inferior vena cava just cephalad to the renal vein was closed with a small bulldog clamp, and the left renal vein immediately entered with a 20-gauge needle through which blood flowed into a citrated one cc. pipette held nearly horizontally. The time required for blood to fill the second 0.5 cc. was measured with a stop watch. The rat was weighed before the final operation; after the experiment it was bled, the kidney and heart weighed, and flow calculated in cc./minute/gram of renal tissue.

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[1] Goldblatt, H., *et al.*, *J. Exp. Med.*, 1934, **59**, 347.

[2] Chanutin, A., and Ferris, E. B., Jr., *Arch. Int. Med.*, 1932, **49**, 767.

[3] Rytand, D. A., and Dock, W., *Arch. Int. Med.*, 1935, **56**, 511.

Results. The averages are shown in Table I.

TABLE I.

	Subtotal Nephrectomy		Unilateral Nephrectomy
	2 weeks post-operative	5 mo. post-operative	5 mo. post-operative
Rate of blood flow, cc./min./gm.	2.14±0.073	1.73±0.096	1.72
Kidney weight, mg./100 sq. cm.	125	177	259
Heart weight, mg./100 sq. cm.	185	210	180
No. of rats	16	14	6

The results show an identical rate of blood flow (1.7 cc./min./gm.) through the renal stump of the hypertensive rat and through the enlarged kidney of the unilaterally nephrectomized rat. On the other hand, flow is significantly increased by 24% (+ 0.41 ± 0.12 cc./min./gm.) through the freshly prepared stump of the non-hypertensive rat at a time when renal hypertrophy is rapid. The kidney stump, at 2 weeks or 5 months, is under a heavy burden as the blood urea is over 200 mg. %

Hayman[4] has perfused excised human kidneys with Ringer's solution under varying heads of pressure. Through normal kidneys and those of uremic patients with chronic glomerulonephritis the flow was more than doubled by raising the perfusion pressure from 100 to 200 mm. Hg. The flow through uremic kidneys, per gram, was only one-third that of normal kidneys at the same perfusion pressure, but as these kidneys consist largely of scar tissue it is not possible to estimate the flow per unit of functioning renal parenchyma. In the present experiments the most rapid rates of renal blood flow were in rats without hypertension, in the early period of renal hypertrophy. Moreover, within the series of hypertensive rats, there was no correlation between the degree of hypertension (as indicated by heart weight per surface area) and the rate of flow. There is greater dilatation of the tubules of the stumps at 5 months and the kidney weights include more inert matter than at 2 weeks, so the observed rates of flow per gram are too low if thought of in terms of active renal tissue. The results really show no significant decrease in blood flow per gram of active kidney tissue as hypertension develops after subtotal nephrectomy. Therefore, if the hypertension is due in some way to a relatively narrow renal vascular bed, its degree is exquisitely adjusted to maintain a normal rate of renal blood flow.

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[4] Hayman, J. M., Jr., *J. Clin. Invest.*, 1929, **8**, 89.

Conclusion. Rats which become hypertensive several months after subtotal nephrectomy do not have renal ischemia; the flow per gram of renal tissue is 19% less than in rats a few days after subtotal nephrectomy, but the same as that in rats with unilateral nephrectomy and without hypertension.

9169

Experimental and Quantitative Analysis of Local Anesthesia of the Frog's Isolated Sciatic Nerve.

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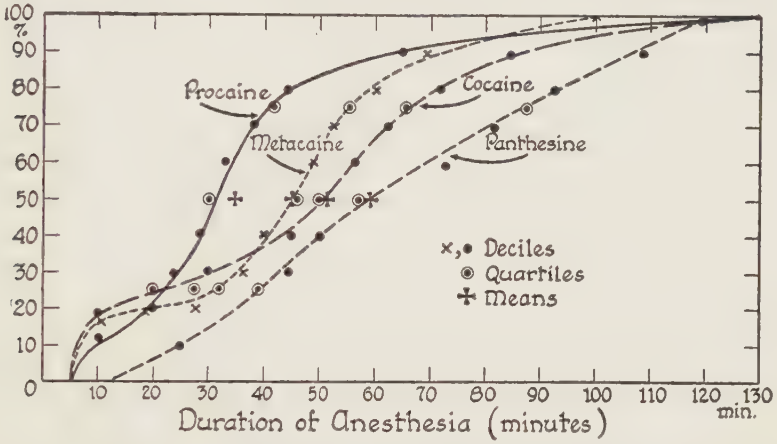
It is a commonly known fact that the characteristic action produced by certain local anesthetic drugs is a somewhat variable function. The extent and behavior of this variation are not well known. In an attempt to describe this variation, quantitative data on the duration of sensory anesthesia in frogs are herein presented. The data were obtained from class experiments of medical students over a period of several years.

The following experimental technique was used. The frogs were single pithed and one sciatic nerve in each frog was exposed in the thigh. The other leg served as a control. The normal reaction of the frog was determined by immersing the toes of one foot in 1% HCl. This causes a rapid withdrawal of this leg from the acid solution. Then the local anesthetic was applied to the isolated sciatic nerve by carefully placing a whisp of absorbent cotton about 3 mm. wide, thoroughly moistened with the local anesthetic solution but not dripping, under and around the nerve. This was allowed to remain in place for exactly 5 minutes. The whisp of cotton was then removed and the toes of the leg so treated were tested in the acid solution every 15 to 30 seconds until sensory anesthesia was produced (that is, when the leg is no longer drawn out of the acid solution during a period of application of 30 seconds). The duration of anesthesia was determined from this time forward by testing the leg every few minutes. It was terminated when the leg was again pulled out of the acid solution as a result of an exposure to



Substance (¼% conc.)	n	Measurses of duration of anesthesia (minutes)					
		Mean & S.E.	Median & S.E.	Semi-inter-quartile range	S.D. & S.E.	C.V. & S.E. %	Skewness
Procaine HCl.	73	34.4 ± 2.2	30 ± 1.9	11	18.9 ± 1.6	54.9 ± 5.8	+0.14
Cocaine HCl.	76	51.9 ± 3.0	50 ± 2.5	18	26.0 ± 2.1	50.4 ± 5.1	-0.22
Metacaine	78	45.1 ± 2.3	44 ± 1.9	12	20.3 ± 1.6	45.0 ± 4.3	-0.13
Panthesine	72	63.3 ± 3.4	59 ± 2.9	24	28.3 ± 2.4	44.7 ± 4.4	+0.18

Substance (¼% conc.)	Deciles & quartiles of duration of anesthesia (min.)												
	(0)	10	20	Q_1 (25)	30	40	Q_2 (50)	60	70	Q_3 (75)	80	90	Q_4 (100)
Procaine HCl.	**6**	12	20	**21**	24	28	**30**	33	38	**42**	44	59	**120**
Cocaine HCl.	**6**	19	25	**28**	30	45	**50**	56	62	**64**	73	85	**130**
Metacaine	**6**	19	25	**31**	36	40	**44**	49	52	**54**	60	69	**95**
Panthesine	**11**	25	35	**39**	43	50	**59**	73	81	**88**	92	102	**120**

Differences between means of duration of anesthesia of pairs of substances				
Substances compared (a–b)	Difference	S.E. of diff.	R	P
Cocaine HCl. (a) — Procaine HCl. (b)	17.2	± 3.7	4.65	0.000,006
Metacaine (a) — Cocaine HCl. (b)	10.7	± 3.2	3.34	0.000,9
Procaine HCl. (a) — Panthesine (b)	28.9	± 4.0	7.20	0.000,000,--
Cocaine HCl. (a) — Metacaine (b)	6.5	± 3.8	1.71	0.087
Panthesine (a) — Cocaine HCl. (b)	11.7	± 4.5	2.60	0.0093
Panthesine (a) — Metacaine (b)	18.2	± 4.1	4.44	0.000,15

FIG. 1.

Ogive graphs and tables showing the measures of central tendency, scatter and comparison of a series of anesthetics applied to the frog's isolated sciatic nerve.

the acid of no longer than 30 seconds. Four local anesthetics were studied, namely, Procaine HCl, Cocaine HCl, Metycaine and Panthesine (each in 0.25% concentration in 0.75% NaCl). From 72 to 78 determinations were obtained on each solution. A separate frog was used for each determination.*

Figure 1 presents our findings in graphic and tabular form. The first panel shows a series of ogives of the distribution of duration, in minutes, of anesthesia by the several substances. In this panel the percentage of observations forms the ordinate and the duration of anesthesia the abscissa. Deciles, quartiles, means and medians are indicated. The curves are drawn by inspection without smoothing.

The second panel gives various measures of central tendency and scatter of duration of anesthesia of the several drugs. All of the measures are the generally accepted ones with the exception of that for skewness. Here we have chosen from the several measures of the quantity the expression suggested by Rietz:[1]

$$\text{Skewness} = \frac{Q_3 + Q_1 - 2Q_2}{Q_3 - Q_1}$$

The third panel gives the quartile, decile and minimum values for each of the drugs studied.

The fourth panel presents tests of the significance of differences of the means of duration of anesthesia of the several drugs. According to commonly accepted conventions, these differences are highly significant for Cocaine HCl—Procaine HCl, Metacaine—Cocaine HCl, Procaine HCl—Panthesine, and Panthesine—Metacaine. The significance of the difference for Panthesine—Cocaine HCl is probably significant and that for Cocaine HCl—Metacaine is very questionable.

Summary. Sensory anesthesia produced by 0.25% concentration in normal salt solution on the frog's sciatic nerve has been tested on a considerable series by immersion of the toes in 1% HCl. The results obtained were: 1. The mean and median durations of anesthesia fall in the following ascending order: Procaine HCl, Metacaine, Cocaine HCl, Panthesine. 2. A high degree of range is found for the 4 drugs studied. 3. The absolute variability in duration of anesthesia falls in the following ascending order: Procaine HCl, Metacaine, Cocaine HCl, Panthesine. 4. The relative variability in duration falls in the following order: Panthesine, Metacaine, Cocaine HCl, Procaine HCl. 5. All distributions of duration of anesthesia show a distinct measure of skewness, those for Procaine HCl and Panthesine being positive and those for Cocaine HCl and Metacaine being negative. 6. The differences in mean duration of anesthesia between the several drugs is highly significant in 4 pairings, probably significant in one, and doubtful in another.

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* Grateful appreciation is accorded to the following firms: The Winthrop Chemical Company, Inc., Eli Lilly and Company, and Sandoz Chemical Works, Inc., for generously supplying the Procaine (Novocain), Metacaine, and Panthesine, respectively, used in these experiments.

[1] *Handbook of Mathematical Statistics* (H. L. Rietz, Editor-in-Chief), Chap. II, p. 31 (Rietz), Boston, 1924.

9170 P

Effects of a Divalent Cation on Sodium Removal from Intestinal Loops.

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It has previously been found that in the presence of any polyvalent anion, the univalent anions such as chloride[1] or bromide[2] move from the intestinal loop into the blood against great concentration differences. The univalent anion may fall to a concentration in the intestine less than 1% of its blood concentration.

The comparable situation has now been tested with respect to cations. A solution containing sodium chloride and magnesium chloride, each in half isotonic quantities, was placed in a washed loop of the lower ileum of the amytal anesthetized dog as previously described.[1] Samples were taken at intervals and sodium determined by the Barber-Kolthoff[3] method, chloride by the Van Slyke[4] technique, and ammonia by the Nash-Benedict[5] procedure.

Figure 1 shows the results of a typical experiment. It is to be noted that the sodium content decreased over a period of 1½ hours, from an initial level which is approximately one-half of the blood plasma level, to 8 mM., which is approximately 5% of the blood plasma level. Meanwhile the chloride content fell to approximately its blood concentration. These results have been obtained uniformly in 10 trials. Other experiments, not reported in full in

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[1] Ingraham, R. C., and Visscher, M. B., *Am. J. Physiol.*, 1936, **114**.

[2] Ingraham, R. C., PROC. SOC. EXP. BIOL. AND MED., 1935, **33**, 453.

[3] Barber, H. H., and Kolthoff, I. M., *J. Am. Chem. Soc.*, 1928, **50**, 1625.

[4] Van Slyke, D. D., *J. Biol. Chem.*, 1923, **58**, 523.

[5] Nash, T. P., and Benedict, S. R., *J. Biol. Chem.*, 1921, **48**, 463.

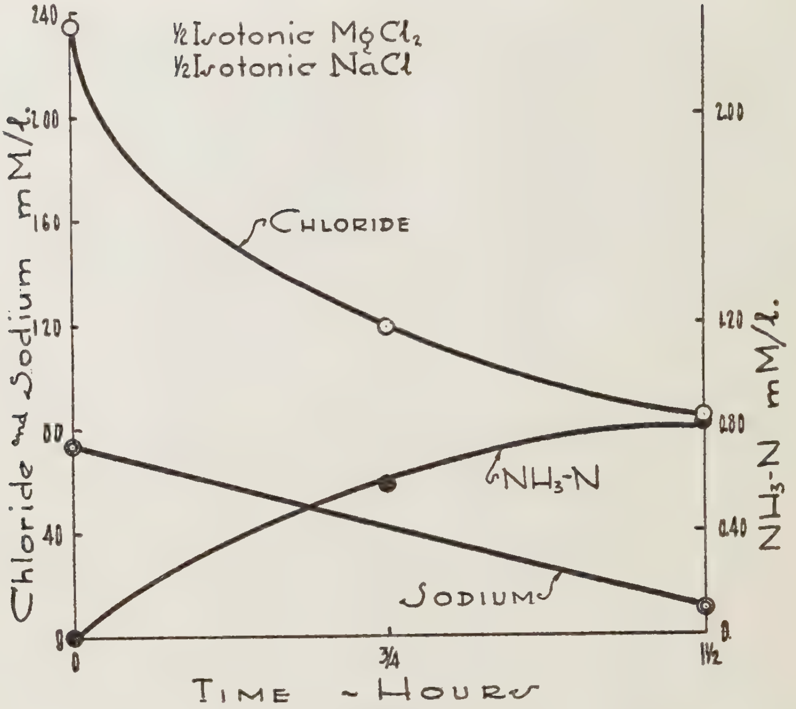

Fig. 1.

this communication, have shown that magnesium accumulates in the gut loop, and that its salts come to approximately isotonic concentration with respect to the total blood osmotic pressure. Certain other polyvalent cations may be substituted for magnesium.

The number of polyvalent cations which can be used to illustrate this phenomenon is limited because of the insolubility of their bicarbonates and other salts at or near neutrality. Aside from magnesium, only the several cobaltamine chlorides have proved useful.

Attention is called to the increase in concentration of ammonia in the intestinal fluid. This is a constant phenomenon in every experiment of this type. Its possible significance will be discussed in another place.

Summary and Conclusion. In the presence of polyvalent cations sodium is absorbed from the intestine from concentrations much lower than the blood sodium level. The sodium concentration in the intestinal fluid may fall to as low as 5% of the blood level. Thus, the previously described generalization that univalent anions move against high concentration gradients from intestine to blood when polyvalent anions are present in the intestine, can be extended to include the comparable situation with regard to cations.

9171

Dissimilation of Pyruvic Acid by *Clostridium butylicum.*

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Clostridium butylicum (Beijerinck) Donker is a member of the butyric acid-butyl alcohol group of bacteria forming large quantities of butyl alcohol; it is characterized by the production of isopropyl alcohol and in this manner is differentiated from *Cl. acetobutylicum,* the organism used in the butyl alcohol-acetone industry. Relatively little work has been done on the metabolism of *Cl. butylicum.* Van der Lek[1] studied the organism and determined the final products.

We have studied the intermediate reactions of cell-suspensions of this species which normally produces butyl and isopropyl alcohols, acetic and butyric acids, carbon dioxide and hydrogen with traces of 2,3-butylene glycol from the fermentation of glucose. Experiments were performed with the idea of determining: (a) the products formed by the action of the cell-suspension on pyruvic acid alone; (b) whether pyruvic acid donates hydrogen for the reduction of butyric acid; (c) whether formic and lactic acids donate hydrogen for the reduction of butyric acid. Pyruvic acid is shown to be an intermediate compound in the breakdown of glucose by *Cl. butylicum;* its behavior becomes of importance in formulating a scheme of dissimilation.

Pyruvic acid was determined by iodoform-titration as given by Wendell,[2] butyl alcohol by the method of Stahly, Osburn and Werkman,[3] butyric and acetic acids according to the method of Osburn, Wood and Werkman,[4] and lactic acid by the Friedmann, Cotonio and Shaffer[5] method. The mercuric oxide method was used for the gravimetric determination of formic acid. Carbon dioxide was absorbed in Bowen potash-bulbs and determined gravimetrically. Hydrogen was collected in bottles by water-displacement and estimated gravimetrically after burning in a combustion-chamber.

Ten liters of broth containing 0.5% yeast-extract, 1% glucose and 0.1% K_2HPO_4 were inoculated with *Cl. butylicum*. After 24 hours' incubation the cells were concentrated to a relatively dry paste by means of the supercentrifuge. Approximately 2 gm. portions of this mass were used in experiments with test substances in the absence of glucose and a source of nitrogen. Under these conditions the bacteria do not multiply but the enzymes are active. The experiments were carried out in 500 cc. Erlenmeyer flasks connected with Bowen potash-bulbs and bottles for collecting the hydrogen. The flasks were made anaërobic by replacing the air with nitrogen, and incubated at 37°C.

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[1] Van der Lek, J. B. (1928), Onderzoekingen over de butylalkoholgisting. Thesis. Delft, Holland.

[2] Wendell, W. B., *J. Biol. Chem.*, 1931, **94**, 717.

[3] Stahly, G. L., Osburn, O. L., and Werkman, C. H., *Analyst.*, 1934, **59**, 319.

[4] Osburn, O. L., Wood, H. G., and Werkman, C. H., *Ind. Eng. Chem. Anal. Ed.*, 1933, **5**, 247.

[5] Friedmann, T. E., Cotonio, M., and Shaffer, P. A., *J. Biol. Chem.*, 1927, **73**, 335.

Pyruvic acid was isolated and identified from 2% glucose-fermentations to which sodium bicarbonate was added to pH 7.0. When the glucose was completely fermented (72 hr.) the medium was cleared with trichloracetic acid and pyruvic acid precipitated as the 2,4-dinitrophenylhydrazone which melted at 212.5°C. Pyruvic acid was further identified as the *p*-nitrophenylhydrazone. We have been unable to isolate pyruvic acid from fermentations conducted in acid media.

Table I shows the results of 2 experiments: (a) pyruvic acid neu-

TABLE I.
Fermentation of Pyruvic Acid.

Product	Millimoles			
	a		b	
	Added	Recovered	Added	Recovered
Pyruvic acid	27.0	1.0	40.0	0.0
Acetic "	—	10.4	—	20.4
Butyric "	—	6.9	—	7.5
CO_2	—	25.7	—	41.5
H_2	—	5.4	—	11.4
Carbon	81.0	77.1	120.0	112.3

TABLE II.
Pyruvic Acid with Butyric Acid.

Product	Millimoles			
	a		b	
	Added	Recovered	Added	Recovered
Pyruvic acid	50.0	0.0	50.0	0.0
Butyric "	10.0	13.5	10.0	13.8
Acetic "	—	21.9	—	24.6
Butyl alcohol	—	9.1	—	13.0
CO_2	—	50.0	—	49.4
H_2	—	8.2	—	8.8
Carbon	190.0	184.2	190.0	205.8

tralized with $CaCO_3$ and the excess carbonate removed by filtration; (b) pyruvic acid adjusted to pH 6.6 with NaOH.

These data show that pyruvic acid was converted to acetic and butyric acids, carbon dioxide and hydrogen. It is of interest that butyric acid under these conditions of pH above 6.3 is not reduced by *Cl. butylicum* to butyl alcohol. The significance of this critical pH will be discussed in a subsequent communication. However, when the pH is less than 6.3, butyric acid is reduced to the alcohol. Table II shows the results from 2 experiments in which a mixture of pyruvic and butyric acids was adjusted to pH 4.8 with NaOH and K_2HPO_4. Although butyric acid was reduced to butyl alcohol, it is to be noted that the reduction was not complete. In experiments with mixtures of formic and butyric acids as well as with lactic and butyric acids no enzymatic activity was manifested. Practically the same molarity of these compounds added was recovered unaltered. It must be considered, however, that the above experimental conditions are somewhat removed from those which obtain in a normal fermentation.

Unpublished results obtained in our laboratory indicate that isopropyl alcohol as well as traces of acetylmethylcarbinol is produced from a mixture of pyruvic acid and acetone. Moreover, lactic and formic acids are formed from pyruvic acid when the pH is maintained slightly above 7.0 by addition of sodium bicarbonate.

Conclusions. Pyruvic acid is converted by cell-suspensions of *Cl. butylicum* into acetic and butyric acids, carbon dioxide and hydrogen. Hydrogen donated by pyruvic acid is available for the reduction of butyric acid to butyl alcohol at a suitable pH. Formic acid it not decarboxylated and lactic acid is not dehydrogenated.

9172

Effects of Androsterone and Testosterone on Oestrous Cycle of Rats.

LUDVIG G. BROWMAN. (Introduced by Carl R. Moore.)

From Hull Zoological Laboratory, The University of Chicago.

Ihrke and D'Amour[1] reported that injections of bull testis-tissue male hormone concentrates prepared by Gallagher and Koch resulted in the suppression of the oestrous cycle of female rats during the period of injections. The cycles, as expressed by the vaginal smear, reappeared when injections were no longer given. These observations and others of a similar nature were interpreted in terms of the hypothesis suggested by Moore and Price.[2] This hypothesis advances possible explanations for the effects produced on the gonads of either sex and their accessories by castration and by the injection of sex hormones and gonadotropic agents.

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[1] Ihrke, I. A., and D'Amour, F. E., *Am. J. Physiol.*, 1931, **96**, 289.

The hypothesis suggests first, that pituitary hormones stimulate the gonads to produce germ cells and to secrete hormones, and second, that the gonad hormones of either sex have a suppressing action upon the pituitary which results in a diminution of pituitary secretions. One effect of such diminution of pituitary secretions is relative inactivity of the gonads. Moore and Price[2] had shown that whereas bull testis extracts prevented or repaired castration damage in accessory reproductive organs of the male rat it had injurious effects upon normal young growing testes, presumably by virtue of its suppressing effect upon the pituitary. Ihrke and D'Amour similarly suggested that bull testis-tissue extracts affected the female pituitary so as to make unavailable to the organism sufficient concentration of pituitary secretions to permit the ovaries to continue their normal gametogenic and hormonal activities.

Because the bull testis-tissue male hormone concentrates employed by Ihrke and D'Amour were impure it was deemed advisable to repeat a portion of their work using pure chemical androgenic agents. At the suggestion of Professor Carl R. Moore a brief study was made of the effects of pure crystalline androsterone and testosterone on the oestrous cycle of female rats. These substances, prepared by synthetic processes, were provided through the courtesy of Dr. C. C. Haskell and the CIBA company, whose generosity is herewith gratefully acknowledged.

Experimental. Sixteen normal female rats, about 4½ months old, showing regular oestrous cycles during a preliminary observation period of 16 days as indicated by twice daily vaginal smears, were selected for the experiment. Fourteen of these females received daily subcutaneous injections of sesame oil solutions, some of which contained the pure androgenic substances. The other 2 females were kept in the same cages with the injected animals as untreated controls. Four concentrations of testosterone in sesame oil were injected into paired females as follows: 2 females each received 0.5 mg. daily for 15 days, two 1 mg., two 2 mg., and two 3 mg. Androsterone in sesame oil was injected into 3 animals in different concentrations: 3, 4, and 5 mg. Three females received injections of sesame oil only in amounts of 0.3, 0.5 and 0.7 cc. These quantities of oil included the range of oil used in carrying the testosterone and androsterone. At the writer's request Professor C. R. Moore presented the oil samples under code number only and their contents were unknown until the termination of the experiment.

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[2] Moore, C. R., and Price, D., *Am. J. Anat.*, 1932, **50**, 13.

The selection of these particular doses was based on the following work. Moore (unpublished) had earlier administered androsterone to normal adult female rats and found that 1 to 2 mg. daily failed to disturb the oestrous cycles, 3 mg. did disturb the cycles, and 4 mg. suppressed them. The present dosage range of androsterone in sesame oil of 3.0, 4.0, and 5.0 mg. was based upon these results in part and also upon the fact that Moore and Price[3] determined that 3 mg. androsterone daily were required to replace the effects of the testis on the growth rate maintenance of the seminal vesicle of young mature castrated male rats. It had further been determined for testosterone that 0.3 mg. daily was insufficient to maintain seminal vesicle growth comparable to that found in normal males. Therefore higher dosages were adopted with a range of 0.5 to 3.0 mg. of testosterone daily. It is assumed that the injection doses should be equal to or greater in androgenic activity than the amount of sex hormone normally produced by the testes if the pituitary gland is to be markedly suppressed.

Results. All of the females receiving injections of testosterone or androsterone were in diestrum by the third day following the first injection and remained so throughout the 16-day period of injections. The 3 females receiving injections of sesame oil alone, and the 2 untreated females, continued their regular cycles undisturbed throughout the experiment. One animal of each pair receiving the 4 different levels of testosterone, one animal receiving androsterone, and 3 controls were killed and their tissues weighed and preserved the day following the last injection. The other 2 controls were killed upon reaching mid-diestrum a day or 2 later. Vaginal smears of the remaining animals showed a return to vaginal cornification within 4 to 8 days following the last injection of androsterone or testosterone.

No significant conclusions were reached from a study of the data obtained on recovery of the internal organs, largely because of their variability and because of the small number of animals involved. Females injected with testosterone had somewhat lighter ovaries and heavier uteri than those of females not injected or injected with sesame oil alone. Animals injected with sesame oil with or without male hormone had lighter pituitaries than normal non-injected controls. Animals injected with sesame oil solutions had an accelerated weight increase during the period of injections over that of their previous weight increase for a similar period just preceding injection, and over that of the normal non-injected controls kept in the same cages.

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[3] Moore, C. R., and Price, D., *Endocrinology*, 1937, in press.

Conclusions. The observed results demonstrate that daily injections of 0.5 to 3 mg. of testosterone in sesame oil and of 3.0 to 5.0 mg. of androsterone in sesame oil into normal mature female rats leads to a suppression of the oestrous cycles, as expressed by the vaginal smear, during the 16-day period of injection.

These observations agree with the findings of Robson[4] that testosterone causes total suppression of the oestrous cycle in mice. It seems quite adequate to the writer to assume that the pure crystalline androgenic substances, testosterone and androsterone, have an action upon the pituitary gland of the female rat in a manner consistent with the interpretation offered by Moore and Price. Nothing presented by these experiments, however, would rule out the possibility of a direct effect upon the ovary by crystalline androsterone or a possible neutralization of the action of estrogenic substances upon the female tract by androsterone.

9173 P

Effect of Various Degrees of Anoxemia on Secretion of Acid and Chlorides by the Stomach.

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Alvarez[1] found that when the hemoglobin fell below 12 gm. per 100 cc. of blood the mean gastric acidity fell sharply and the incidence of achlorhydria rose rapidly. Apperly and Cary[2] showed that when the red cell count of the blood fell below a certain critical level, free acid disappeared from the stomach. The acidity was also found to decrease with an increase in the erythrocyte count above a certain normal level. It was felt that a study of gastric secretion under a condition of anoxemia might be of value in understanding the relation between the blood and gastric acidity.

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[4] Robson, J. M., Proc. Soc. Exp. Biol. and Med., 1936, **35**, 49.

[1] Alvarez, W. C., and Vanzant, F. R., *Proc. of the Staff Meetings of the Mayo Clinic*, 1936, **11**, 385.

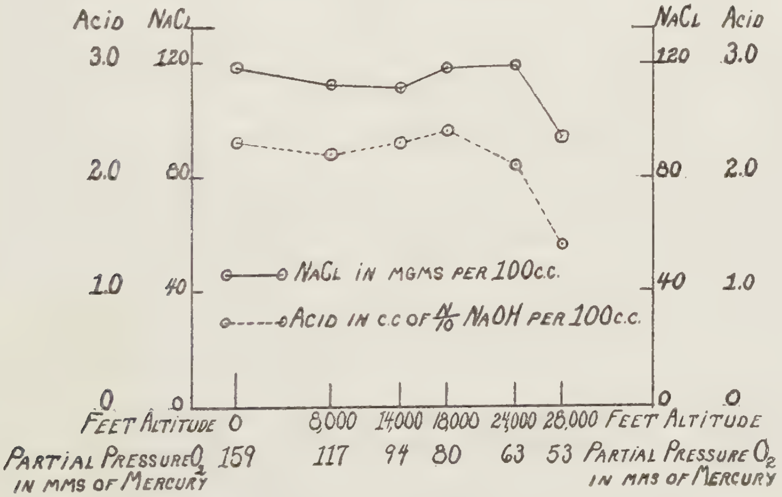
[2] Apperly, F. L., and Cary, M. K., *Am. J. Dig. Dis. and Nutr.*, 1936, **3**, 466.

Barbitalized dogs and cats (220 mg. per kilo), starved for 48 hours previous, were used. Two animals of approximately the same weight were chosen, one to serve as a control, the other to be subjected to anoxemia. The stomach was ligated at each end, washed, and filled with a measured amount of distilled water at body temperature. After 3 hours the stomach was removed and its contents measured and analyzed for acid and chlorides. During the 3-hour period the experimental animal was placed in a respiratory chamber and subjected to the desired degree of anoxemia. Partial pressures of oxygen of 117, 94, 80, 63 and 53 mm. of mercury were used.

Table I and Chart 1 show the results obtained. It will be seen

TABLE I.

Altitude in feet	Partial Pressure Oxygen Mm. Hg.	No. animals	Total Acidity cc. of N/10 NaOH per 100 cc.	Chloride secreted Mg. NaCl/100 cc.
0	152	74	2.3	118
8,000	117	4	2.2	112
14,000	94	19	2.3	111
18,000	80	20	2.4	118
24,000	63	14	2.1	119
28,000	53	17	1.4	94



THE EFFECT OF ANOXEMIA ON GASTRIC SECRETION

CHART 1.

that at less severe degrees of anoxemia both the acid and chloride secretion approach normal very closely. At a partial pressure of oxygen of 53 mm. of mercury (28,000 feet), a very severe degree of anoxemia, the secretion is appreciably depressed.

Anoxia is one of the concomitants of severe anemia. Wright[3] shows that in severe anemia the amount of hemoglobin may be so small that only half the normal amount of oxygen can be present, although there is 95% hemoglobin saturation. In anoxia, the type produced in this work, the oxygen content of the blood remains nearly normal until the oxygen tension falls so low that hemoglobin unsaturation is considerable. This probably explains why less severe degrees of anemic anoxia are capable of depressing gastric secretion more than is anoxic anoxia.

Koehler, *et al.*,[4] have shown that the degree of anoxic anoxia, which we have found capable of depressing gastric secretion produces a severe acidosis, probably incompatible with life for more than a few hours.

We feel that polycythemia could be ruled out as a causative factor because of the short duration of the experimental period.

It is believed that the anoxemia itself is responsible for the depression of secretion seen at the most severe degree. This may be due to actual or incipient tissue asphyxia, disturbance of the acid-base balance, or some other factor.

The fact that the secretory function of the stomach is capable of withstanding anoxemia so well is not entirely in accord with the statement of Hellebrandt[5] that the secretory and motor functions of the stomach run parallel. It has been shown that comparatively moderate degrees of anoxemia depress gastric motility in both the empty and the full stomach.[6, 7]

Summary. It was found, in barbitalized dogs and cats, that anoxemia less severe than that produced by breathing air with a partial pressure of oxygen of 53 mm. of mercury had no effect on gastric secretion. When this partial pressure, a very severe degree of anoxemia, is reached there is an appreciable depression of the secretion of acid and chlorides by the stomach. One may thus conclude that gastric secretion in response to filling the stomachs of barbitalized animals with water is appreciably influenced only by very severe degrees of anoxemia. It also seems that anemic anoxia is more effective in depressing gastric secretion than is anoxic anoxia.

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[3] Wright, Samson, *Applied Physiology*, Oxford University Press, 1935, p. 418 ff.

[4] Koehler, A. E., Behneman, H. M. F., Bennell, O. E., and Loevenhart, A. S., *Am. J. Physiol.*, 1925, **74**, 590.

[5] Hellebrandt, F. A., and Dimmitt, L. L., *Am. J. Physiol.*, 1934, **107**, 364.

[6] Van Liere, E. J., and Crisler, George, *Am. J. Physiol.*, 1930, **93**, 267.

[7] Van Liere, E. J., Crisler, George, and Robinson, Dennis, *Arch. Int. Med.*, 1933, **51**, 796.

9174 P

Studies on Zinc.

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Young rats weighing between 40 and 50 gm. were placed in groups of 5, three females and 2 males, in screen bottom cages. Food and water were supplied *ad libitum.* C. P. zinc carbonate was incorporated in the growing ration at 3 levels, corresponding to 0.10, 0.50, and 1.00% of zinc. The growing ration is a mixture of naturally occurring foods, and has served this laboratory well, during many years, for the growth and reproduction of rats. The zinc salt contained as a maximum the following impurities: arsenic 0.000%, chloride 0.002%, iron 0.01%, lead 0.002%, nitrate 0.000%, and sulphate 0.01%. Hemoglobin was determined by the Newcomer method.

Growth was normal on the rations containing 0.10 and 0.50% of zinc. The animals were vigorous when the experiment terminated, and there was practically no difference in the weight of the rats receiving zinc and those on the stock diet. The limit of tolerance to zinc carbonate lies between 0.50 and 1.0% of zinc in the diet. The animals on the 1% zinc diet failed to grow in the majority of cases, although some individuals weighed 140 to 160 gm. after 10 to 12 weeks. Some of the animals began dying within 4 weeks after being placed on this diet; some were partly eaten.

Growth and reproduction on the lowest level of zinc were normal. Several generations of these rats on the same zinc diet have shown no abnormalities. Reproduction was markedly affected, when the animals received 0.50% of zinc. The females on this diet became pregnant between the fourteenth and seventeenth weeks. One female had 3 young and lost 30 gm. at parturition. Another female had 3 young (one born dead) but lost 40 gm. at parturition. Another female when observed had 5 dead young weighing 24 gm. The young appeared white and bloodless. The females with the live young mothered them satisfactorily. The young averaged 30 gm.

at 21 days of age. The original females again mated when returned to their cages. The young were dead in every case and no live young were born after the first pregnancy. A total of 23 still born rats were counted and others were undoubtedly consumed by the mothers. The females on 0.50% zinc ceased to become pregnant after 5 months on the ration, although their weights and outward appearances were normal. No reproduction occurred on the 1% zinc level.

TABLE I.
Effect of Zinc Carbonate on Hemoglobin and Red Cells of the Rat.

Lot No.	Zinc Intake %	Initial Hemoglobin gm. per 100 cc.	Final Hemoglobin gm. per 100 cc.	Initial R.B.C. millions per cu.mm.	Final R.B.C. millions per cu. mm.	Weeks on Ration
1	0.10	14.5	14.7	8.4	9.1	39
2	0.50	13.9	10.2	8.6	8.5	39
3	1.00	14.2	6.1	8.5	6.6	12-16
4	1.00	13.6	6.5	7.8	6.5	10-16
5	1.00	13.7	6.8	8.0	6.8	12-16
6	1.00	13.5	7.8	8.1	5.8	10
7	0.00	14.1	14.1	7.8	8.9	34
8	0.00	14.6	15.1	—	—	30

Table I shows that normal hemoglobin values were found in the group of rats receiving 0.10% of zinc after 39 weeks on this diet. That there was something organically wrong with the animals on the 0.50% zinc level was first made evident from observations on their reproductive behavior. At the age of initial reproduction of these females the hemoglobin values were only slightly lower than those of the control group. They were still within the limits given by some investigators as normal, but when the rats were kept longer on the ration they became anemic. The average hemoglobin value at 30 weeks was 10.2 gm. No change in the number of R.B.C. was observed. Both hemoglobin and red blood corpuscles were diminished on the 1% zinc diet. Hemoglobin values in this lot usually started to decrease after 3 to 5 weeks on the ration. Low values of 4 gm. % were sometimes observed. Rats often lived for 5 weeks with hemoglobin values below 6 gm. %, while others succumbed with the hemoglobin only slightly below normal. The blood cell picture of the animals on 1% of zinc was interesting. Although the red cells became irregular in size and shape within 5 weeks after being placed on this diet, the number was not appreciably diminished until after 8 to 10 weeks on this regime. There was apparently an excess of white and immature red blood corpuscles in every case.

Normal females gave average blood hemoglobin values at 7 days and one day before parturition of 14.6 and 12.6 gm. per 100 cc. respectively. At parturition and one week after, the corresponding values were 12.26 and 14.2. We believe that this normal anemic condition is so increased by zinc, when ingested at a 0.50% level, that normal reproduction is impossible, although growth is not impaired. Hemoglobin and reproduction became normal in the animals on 0.50% zinc, when the salt was removed from the ration. The same is true of the hemoglobin of rats on 1% zinc; whether reproduction is permanently impaired on the highest level of zinc is not known.

9175

Rôle of Pituitary in Effect of Testosterone on the Mammary Gland.

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It has been found that the administration of testosterone causes development and secretion of the mammary gland both in the male and in the female normal or castrate rat[1] and similar findings have more recently been reported by Nelson and Gallagher[2] following treatment with Androstene-diol and Androstene-dione. It appears that the trophic action of the testis hormone on the mammary gland has physiological significance, for the mamma of male rats is normally fairly well developed at any time after puberty and shows atrophy following the removal of the testis.[3] Since oestrin has a similar stimulating effect on the growth of the mammary gland in normal or castrate male or female rats, but has no effect in the absence of the hypophysis,[4, 5] it appeared of interest to establish whether the presence of the pituitary is also essential for the mammary gland stimulating effect of testosterone.

Nine male rats, 38 to 42 days of age, were hypophysectomized.

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[1] Selye, H., McEuen, C. S., and Collip, J. B., Proc. Soc. Exp. Biol. and Med., 1936, **34**, 201.

[2] Nelson, W. O., and Gallagher, T. F., *Science*, 1936, **84**, 230.

[3] McEuen, C. S., Selye, H., and Collip, J. B., Proc. Soc. Exp. Biol. and Med., 1936, **34**, 56.

[4] Selye, H., Collip, J. B., and Thomson, D. L., Proc. Soc. Exp. Biol. and Med., 1935, **32**, 1377.

[5] Selye, H., and Collip, J. B., *Endocrinol.*, 1936, **20**, 667.

Four of them received 200 γ of testosterone* in oil subcutaneously daily beginning treatment immediately after operation, while in the remaining 5, treatment with the same dose was initiated 21 days after the operation so as to leave time for atrophy of the mammary gland to occur. In both these groups, treatment was continued for 19 days. At that time histological examination of the mammary glands showed no stimulation under the effect of testosterone, while similar treatment invariably caused growth and development of the mamma in normals.

In another group of 11 hypophysectomized male rats of the same age as the previous group, 100 units of anterior pituitary-like hormone of pregnancy urine were given subcutaneously during a period of 19 days. In 6 animals of this group, treatment was initiated immediately following the operation, and in 5 an intermediate period of 21 days was allowed. At autopsy on the 19th day of treatment, only very slight development of the mammary gland was observed in the group in which treatment was initiated immediately (probably due to incomplete involution) and no development was observed in the other group. The interstitial tissue of the testis showed marked development in both A.P.L. treated groups, the seminal vesicles and prostates were hypertrophic, but the seminal epithelium showed atrophy. It appears from these experiments that testosterone, whether it is injected directly in the form of the synthetic product or whether it is liberated from the testis under the influence of A.P.L., has no effect on the development of the mammary tissue, for the proliferation of which a pituitary stimulus is essential.

This conclusion receives further support from a third series of 10 hypophysectomized male rats of the same age as the previous groups receiving daily subcutaneous injections of one cc. of an anterior pituitary gonadotropic extract.† Six animals received treatment immediately after operation and 4 after a period of 21 days. In both groups the testis was found normal and the mammary gland developed to the extent to which it usually develops in normal male rats when examined on the 19th day of treatment.

The weight of the testis was not appreciably influenced by the testosterone treatment in either group. The average was 505 mg. in untreated hypophysectomized rats 19 days after operation and 552 mg. in testosterone-treated ones in which treatment was begun immediately after the operation and continued for 19 days. It was 264 mg. in untreated controls on the 40th day after operation as compared with 285 mg. in the group in which testosterone was given for 19 days after an intermediate period of 21 days between the operation and the initiation of treatment. This is of interest in connection with the publication of Walsh, *et al.*,[6] and Nelson and Gallagher,[2] who conclude that hypophysectomized rats receiving "male sex hormone" prepared from urine show no testicular atrophy and could not be distinguished from the normal even histologically.

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* The authors are indebted to Dr. Schwenk of the Schering-Kahlbaum Company for a generous supply of testosterone.

† The strength of this extract was such that 1/40 cc. given thrice daily for 3 days enlarged the ovaries of immature rats to an average weight of 50 mg.

Summary. Testosterone, which causes mammary gland development in normal rats, has no effect on the mamma after hypophysectomy. The maintenance of the normal testis structure which has been obtained in hypophysectomized rats by the administration of male sex hormone preparations prepared from human urine is not due to testosterone, for the synthetic product does not prevent testis atrophy.

9176 P

Phospholipids as Oxygen Carriers.

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One of the early hypotheses[1, 2] regarding the function of the phospholipids was that they took part in the oxidation processes of the living cell, the belief being based on their well-known ability to take up oxygen. It was assumed, without however any evidence to support the assumption, that the oxidized phospholipids could act as oxidizing agents or oxidases, passing on their oxygen to other substances in the living cell.

The ability of the phospholipids to take up oxygen under approximately physiological conditions has been satisfactorily established.[3-6]

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[6] Walsh, E. L., Cuyler, W. K., and McCullagh, D. R., *Am. J. Physiol.*, 1934, **107**, 508.

[1] Koch, W., *Z. physiol. Chem.*, 1903, **37**, 181.

[2] Fränkel, S., and Dimitz, L., *Wien. klin. Wochschr.*, 1909, **22**, 1777.

[3] Meyerhof, O., *Arch. ges. Physiol.*, 1923, **199**, 531.

[4] Hopkins, F. G., *Biochem. J.*, 1925, **19**, 787.

[5] Tait, H., and King, E. J., *Biochem. J.*, 1936, **30**, 285.

[6] Page, I. H., and Bülow, M., *Z. physiol. Chem.*, 1935, **231**, 10.

The present experiment was directed at the second part of the oxidase hypothesis, *i. e.,* to find out whether the oxidized phospholipids could supply oxygen for the oxidation of other substances. The plan of the experiment was as follows: Reduced methylene blue was subjected under anaerobic conditions to the action of suspensions of purified liver and muscle phospholipids in buffers at various pH, and the resulting color production determined. A typical experiment was as follows:

Reduced Methylene Blue. 0.001M methylene blue was reduced by dextrose in hot, faintly alkaline solution in a current of nitrogen (40 cc. of methylene blue, 1-2 drops of 40% alkali, 2-5 drops of 5% dextrose). After centrifugation, the colorless samples were drawn with a fine tube from near the bottom of the solution.

Phospholipid Suspensions. Prepared by dissolving 10 mg. of phospholipid in 10 cc. of the different buffer solutions by bubbling a stream of nitrogen through. A control of buffer alone in a matched tube was similarly prepared. After replacement of the air with nitrogen, both tubes were tightly stoppered.

For the experiment, 2 cc. of the reduced methylene blue were introduced into each tube and mixed with a stream of nitrogen. Comparison of the color produced was made in a photoelectric colorimeter similar to that described by Evelyn[7] using a suitable light filter. Typical experiments resulted as shown in Table I.

TABLE I.
Unoxidized Phospholipid.

	Readings	Absorption
	mm.	
Buffer alone	200	
,, + phospholipid	150	50
,, + red. m. blue	148	52
Total absorption by reagents		102
Buffer + phospholipid + red. m. blue	100	108
Difference in absorption due to oxidation		+6

This difference is within the limit of error of the experiment and therefore inconclusive.

Determinations were made at pH 5, 6, 7, and 8 with purified phospholipids (lecithin and cephalin) from liver and muscle, with no results to indicate oxidation of the reduced methylene blue.

Oxidized Phospholipid. The purified phospholipid dissolved in petroleum ether was distributed from a pipette on large sheets of filter paper. After evaporation of the petroleum ether, the sheets were exposed in the laboratory for 48 hours, after which the oxidized phospholipid was extracted by ether and the experiments carried out as before. The material had now markedly oxidizing properties. Moreover it was soluble in water to a clear solution giving a phospholipid + buffer solution which transmitted light without absorption. A typical reading (liver cephalin 10 mg.) is given in Table II.

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[7] Evelyn, K. A., *J. Biol. Chem.,* 1936, **115**, 63.

TABLE II.
Oxidized Phospholipid.

	Readings	Absorption
	mm.	
Buffer alone	200	
,, + phospholipid	200	0
,, + red. m. blue	115	85
Total absorption by reagent		85
Buffer + phospholipid + red. m. blue	28	172
Difference in absorption due to oxidation		+87

Lecithin gave similar but somewhat lower values. As before, the pH of the solution in the range 5 to 8 had no effect.

Summary. A buffered suspension of the phospholipid from liver and muscle had little or no oxidizing effect on a sensitive preparation of reduced methylene blue. After exposure to air for 24-48 hours, the phospholipid became soluble in water to a clear solution and had marked oxidizing effect on the reduced methylene blue. pH changes of from 5 to 8 were found not to affect the oxidation.

9177 P

Growth-Factors for Propionic and Lactic Acid Bacteria.

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Wood, *et al.*,[1] and Tatum, *et al.*,[2] showed that an ether-soluble acid (obtained from yeast-extract, potato-extract or corn-steep) and vitamin B_1 stimulate growth of the propionic acid bacteria. Snell, *et al.*,[3] have shown that ether-extract of potato stimulates *Lactobacillus delbrückii* but an additional factor present in peptone is essential for their growth in hydrolyzed casein, tryptophane and glucose medium.

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* Journal Paper No. J. 440 of the Iowa Agricultural Experiment Station. Project No. 421.

[1] Wood, H. G., Tatum, E. L., and Peterson, W. H., *J. Bact.*, 1937, **33**, 227.

[2] Tatum, E. L., Wood, H. G., ad Peterson, W. H., *Biochem. J.*, 1936, **30**, 1898.

[3] Snell, E. E., Tatum, E. L., and Peterson, W. H., *J. Bact.*, 1937, **33**, 207.

In the present investigation we have continued this study, particularly in determining the effect of lactoflavin on growth. Lava, *et al.*,[4] fractionated yeast-extract and found the portion which contained B_2 was the most active. They suggested that flavin or B_2 might be a stimulant for the propionic acid bacteria but were unable to prove this because of the impurity of their B_2 preparation and lack of a basal medium free of B_2.

We have tested the activity of lactoflavin by adding it to a basal medium which contained per 100 cc. (1) ether-extract of 3 gm. of yeast-extract, (2) 1.0 gamma B_1 (Merck's crystalline), (3) 0.3 gm. $(NH_4)_2SO_4$, (4) 0.6 gm. NaOAc, (5) 1.0 gm. glucose, (6) inorganic salts. In this medium most propionic acid bacteria failed to grow after the 4th transfer. Addition of hydrolyzed casein or a mixture of purified amino acids gave consistently good growth throughout numerous transfers with all cultures tested (11W, 34W, 39W, 52W, 53W, 49W).

Crystalline lactoflavin was added to the basal medium in concentration equivalent to 0.00, 0.01, 0.05, and 0.20 mg. per 100 cc. of medium; 5 cc. amounts of medium were inoculated with one drop of a suspension of cells grown on yeast-extract medium. Transfers were made after 3 days' growth at 30°C. and the acid was titrated after incubation for 5 days. The stimulation produced by lactoflavin was evident on the first transfer but by repeated transfer the results were more distinct. With lactoflavin there was a shorter lag phase. Culture 34W produced the following quantities of N acid per 100 cc. of medium when no flavin was added, 2.2, 1.9, 2.3, 0.8, 1.2, 0.4, 0.9 and with 0.1 mg. of flavin, 3.6, 3.4, 4.4, 3.1, 4.0, 4.1, 4.4 respectively on the 1st, 2nd, 3rd, 4th, 5th, 6th, 7th transfers. Certain cultures failed to grow on repeated transfers and apparently require amino acids even in the presence of lactoflavin which is non-essential when amino acids are supplied.

The lactic acid cultures were not able to grow on the basal $(NH_4)_2SO_4$ medium excepting one unidentified homofermentative-lactic strain. It does not require amino acids or lactoflavin but ether-extract of yeast-extract is indispensable for its growth on the medium and vitamin B_1 acts as a further stimulant. Its simple growth-requirements imply that it may be of considerable economic importance.

With other homofermentative-lactic bacteria, *L. delbrückii* and

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[4] Lava, V. G., Ross, R., and Blanchard, K. C., *Philippine J. Sci.*, 1936, **59**, 493.

L. helveticum, no growth has been observed when hydrolyzed casein, tryptophane and lactoflavin are added to the basal medium. Addition of an alcohol-soluble fraction from malt-sprouts stimulates growth.

The heterofermentative-lactic bacteria are somewhat different in their requirements. If 0.15 gm. of hydrolyzed purified casein is added to 100 cc. of the basal medium, culture L4, *L. pentoaceticus,* does not grow vigorously but when tryptophane (10 mg.) is added as well, growth is abundant on repeated transfer. Tryptophane is undoubtedly a beneficial if not an essential amino acid for *L. pentoaceticus.* Snell, *et al.*,[3] have shown the same to be true for certain homofermentative-lactic bacteria but Orla-Jensen, *et al.*,[5] state that tryptophane is not essential for the lactic acid bacteria. Since their basal medium contained whey, it is doubtful whether the conclusion is justifiable. When purified amino acids (glycine, alanine, valine, leucine, isoleucine, phenylalanine, tyrosine, cystine, methionine, tryptophane, proline, hydroxy-proline, aspartic acid, glutamic acid, arginine, lysine, histidine) were substituted for the hydrolyzed casein, growth was very poor. Apparently some additional amino acid is essential. Cultures L2 *L. mannitopoeus,* L5 *L. lycopersici,* and S9 *Streptococcus paracitrovorous* grow very poorly on the basal medium plus hydrolyzed casein and tryptophane but when lactoflavin is added the growth is more luxuriant and the cultures may be carried through repeated transfer. This observation of the activity of lactoflavin in lactic fermentation is similar to that made by Orla-Jensen, *et al.*[5] Since pantothentic acid is ether insoluble, their conclusion that it is essential to the lactic acid bacteria is questionable. The only unknown factor in our medium other than hydrolyzed casein is the ether-soluble factor.

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[5] Orla-Jensen, S., Otle, N. C., and Snog-Kjer, Agnete, *Kgl. Danske Vidensk. Selskav,* Copenhagen, 1936, ser. 9, **6**, 1.

9178

Effect of Rate of Freezing on the Transmitting Agent of Neoplasms of Mice.*

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Procedures involving the exposure of viruses and mammalian tumor cells to low temperature are widely used in experimental investigations, yet very few systematic studies concerning the effects of freezing and thawing on mammalian tissues have been reported, and the results are contradictory (Cramer[1]). Several factors are involved, among them the rate at which freezing and thawing are accomplished and the actual temperature to which the tissues are exposed. It is generally believed that normal cells are killed by freezing at a certain critical temperature, but this temperature has never been precisely determined for various types of mammalian cells. The studies of Moran[2] indicate that there is a critical temperature, approximately –2°C., at which freezing irreversibly damages muscle tissue of the frog. Lake[3] failed in attempts to cultivate *in vitro* fetal heart tissue of the rabbit that had been submitted to a temperature below –7°C. during 30 minutes. Cramer[1] observed no live cells in tissue cultures of sarcomata or carcinomata which had been subjected 3 times to repeated freezing and thawing, at a temperature of from –20°C. to –40°C.

Previous experiments performed by us[4, 5, 6] with malignant myeloid and lymphoid tissue of mice suggested that the transmitting agent perished when exposed to from –13° to –30°C. and kept at that temperature during 30 minutes. In these experiments the tissue was frozen rapidly. Rapid freezing, which is associated with the formation of small crystals, is generally believed to be less injurious than slow freezing, which is associated with the formation of large crystals. When we found that a leukemic tissue, inadvertently

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* These investigations have been supported by grants from the International Cancer Research Foundation and the Lady Tata Memorial Trust, and by a Fund for the Study of Leukemia.

[1] Cramer, W., *Ninth Scientific Report of the Imperial Cancer Research Fund*, London, 1930, 21.

[2] Moran, T., *Roy. Soc. Proc. B.*, 1930, **65**, 180.

[3] Lake, N. C., *Lancet*, 1917, **2**, 557.

[4] Furth, J., Seibold, H. R., and Rathbone, R. R., *Am. J. Cancer*, 1933, **19**, 521.

[5] Furth, J., *J. Exp. Med.*, 1935, **61**, 423.

[6] Barnes, W. A., and Furth, J., Proc. Soc. Exp. Biol. and Med., 1935, **33**, 386.

frozen slowly, retained the ability to produce leukemia, we began systematic investigations on the rôle of the rate of freezing. Table I summarizes the results obtained with the cells of leukemia Strain S2.

TABLE I.
Effect of the Rate of Freezing on the Agent Transmitting Leukemia.

Freezing					Successful injections	
Rate	Temperature °C.	Duration min.	No. of experiments	No. of mice injected	No.	%
Rapid	—30	30	3	12	0	0
”	—30	180	1	5	0	
Slow	—30	30	3	13	11	76
”	—30	180	1	4	4	
”	—30	420	1	4	2	
”	—70	30	1	4	2	
Not frozen	—	—	3	10	9	90

Rapid freezing was accomplished by suddenly immersing the sealed test tube containing the leukemic cells suspended in Tyrode solution into ether chilled to the desired temperature by solid carbon dioxide. Gradual reduction of the temperature of the ether by dropping into it small pieces of solid CO_2 during a period of from 10 to 20 minutes constituted slow freezing. Thawing was made slowly by keeping the sealed tubes containing the frozen cell suspension first at refrigerator and subsequently at room temperature.

Table I indicates that the agent transmitting leukemia of mice survives exposure to –70°C. during 30 minutes if the temperature is reduced slowly, but is destroyed if exposed rapidly to –30°C. and kept at this temperature during 30 minutes.

The duration of exposure is not the major factor in determining the survival of the agent. This is indicated by experiments in which leukemic tissue, slowly frozen to –30°C. during 180 minutes, produced disease in mice fatal after approximately the same number of days as the disease produced by tissue exposed for 30 minutes only (Table II). Since this time interval was approximately the same in the 2 groups of mice injected with slowly frozen material kept at –30°C. during 30 and 180 minutes respectively, it may be assumed that the same number of viable cells was introduced. Previous experiments[5, 7, 8] have shown that the length of life of mice after inoculation with leukemic cell suspensions is inversely related to the number of viable cells injected.

In one experiment not shown in the table, leukemic tumor tissue

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[7] Richter, M. N., and MacDowell, *J. Exp. Med.*, 1932, **57**, 1.

[8] Unpublished data of the authors.

TABLE II.

Effect of the Duration of Freezing on the Transmitting Agent of Leukemia.

Freezing				Length of life after inoculation	
Temperature °C.	Duration of exposure min.	No. of mice injected	No. of successful injections	Average days	Extremes days
—30	30	4	4	22	19-26
—30	180	4	4	19.2	18-20
Not frozen	—	2	2	12	12

was frozen very slowly, gradually reaching a temperature of –70°C. Eleven of 12 mice injected with tissues kept at this temperature during 10 days developed progressively growing tumors at the site of inoculation.

Determinations of the actual temperature of the tissues made with the aid of thermocouples have shown that the temperature of tissues enclosed in a sealed test tube and rapidly frozen by immersion in an ether bath approximates the temperature of the ether bath within 60 seconds. The temperature of an alcohol bath is taken up more slowly (Chart 1). These measurements were made with the advice of Dr. J. D. Hardy of the Russell Sage Institute of Pathology.

One couple was immersed in the bath and the other was embedded in the center of the tissue inside of a test tube sealed with wax. The inside diameter of the test tube was 9 mm., the wall thickness 0.5 mm. The temperature of the bath was measured with a pentane thermometer. The temperature difference between the 2 couples was determined from the readings of a galvanometer connected to them. Readings were taken until the galvanometer showed no deflection. The temperature difference between the tissue and the bath after this time was less than 0.2°C. In the experiments in which the tissues were used for inoculations, the tubes were sealed off with a blast lamp before freezing.

Chart 1 shows the temperature drop of tissues sealed in test tubes, at different intervals after submersion in ether at –30°C. and at –70°C., and in alcohol at –30°C.

Sarcoma tissue of mice exposed to –70°C. and kept at that temperature for 3 days, was still able to produce progressively growing tumors in inoculated mice. The following experiment shows that in the frozen state deterioration of the agent transmitting sarcoma of mice is slight.

Six mice were injected with cut-up tumor particles which had been frozen at –70°C. for 3 days. Progressively growing tumors appeared in all of these mice approximately 12 days following inoculation. The injection of tumor tissue kept frozen at the same tem-

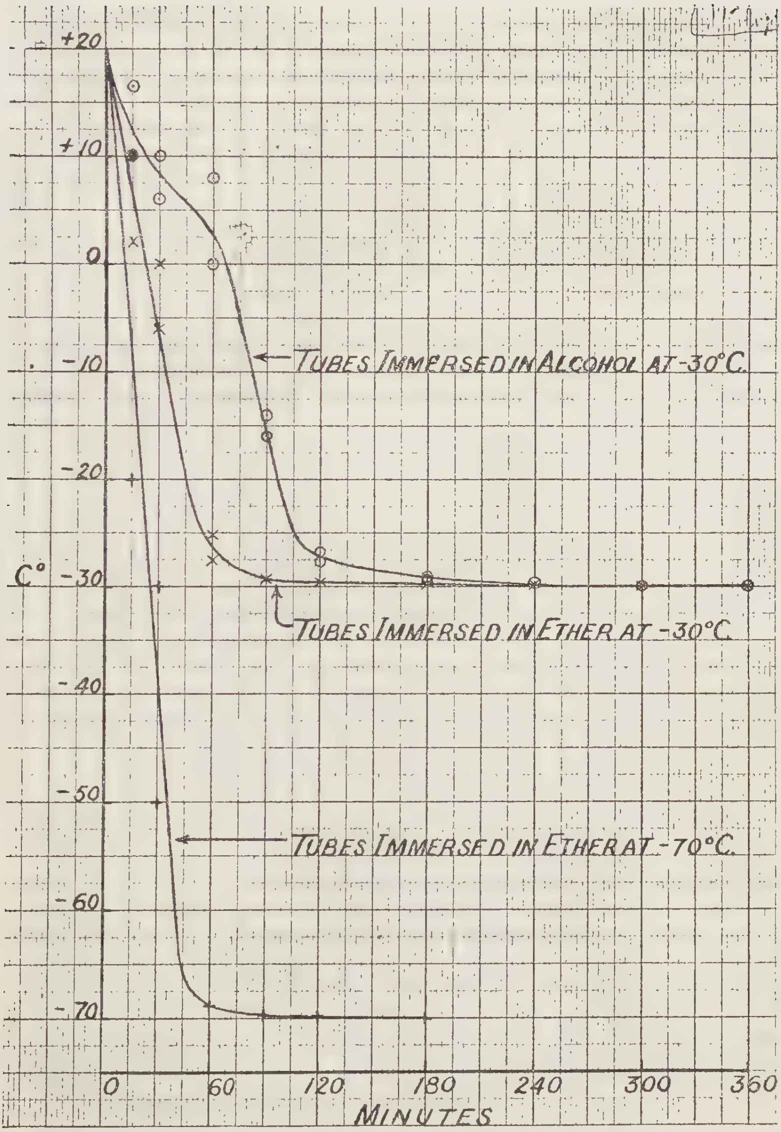

CHART 1.

perature for 56 days was followed by the appearance of tumors in 3 of 4 injected mice, after approximately the same time as in the previous group of mice. The growths that appeared in the mice injected with frozen tumor tissue were similar to those in the mice that had received the fresh material.

Since this experiment indicates that there is very little deterioration of the transmitting agent of this tumor at –70°C. during 56 days, it is highly probable that the agent will remain viable at this temperature for several years.

The ability of frozen and thawed malignant cells to produce tumors has been noted by several workers (Cramer[1]), and the suggestion has been made that the production of the disease by frozen material may be due to the presence of a virus in the mammalian growth. This assumption has been supported by the failure of Cramer to cultivate cells *in vitro* from tumor material that had been subjected to freezing and thawing. His findings are in agreement with ours, yet the following observations indicate that the production of tumors by frozen tissues is due to the survival of a few neoplastic cells. If frozen slowly the malignant leukocytes and sarcoma cells retain their ability to produce tumors at the site of injection, and the growths are composed of cells indistinguishable from those that were introduced. If mice are killed while the subcutaneous leukemic tumors produced by frozen material are small, the blood forming organs exhibit no leukemic alterations. Moreover, such leukemic tumors are more readily produced in mice whose blood-forming tissues have been injured by X-rays prior to inoculation. Furthermore, attempts to transmit leukemia or tumors of mice by cell-free filtrates, glycerinated material, and material dried *in vacuo* in the frozen state, have been uniformly unsuccessful.[6, 8]

These findings indicate that transmission of leukemia and tumors by frozen tissue is due to survival of a few neoplastic cells, but the viability of these cells has thus far not been demonstrated by direct methods.

Many of the problems connected with the effect of freezing on mammalian cells remain to be studied. We are still ignorant of the rôle of the rate of thawing and of the critical temperature at which various types of normal and malignant cells perish. In all of three recent experiments slowly frozen and rapidly thawed material produced tumors in more animals and after a shorter incubation period than material slowly frozen and slowly thawed.

Summary. The transmitting agent of leukemia of mice, presumably malignant leukocytes, is inactivated by rapid freezing to –30°C., but remains viable even at –70°C. when frozen slowly. Sarcoma tissue of mice can also be frozen to at least –70°C. without being inactivated, and can be preserved at this temperature with little or no subsequent deterioration during at least 56 days.

9179 P

Efficacy of *p*-Aminobenzenesulfonamide in Experimental Type III Pneumococcus Pneumonia of Rats.

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Data presented in a preliminary paper[1] indicated prolongation of life, and in some cases complete recovery, of mice which had been infected subcutaneously with 1 to 10 M.L.D.-s of Type III pneumococci and then treated orally with *p*-aminobenzenesulfonamide. Shortly before this work went to press, Rosenthal[2] reported similar protection against infections with Types I, II, and III pneumococci in mice.

Inasmuch as experimental pneumonia resembling human lobar pneumonia has been produced in rats infected intrabronchially,[3, 4] it appeared that results obtained in such rats by the sulfonamide ther-

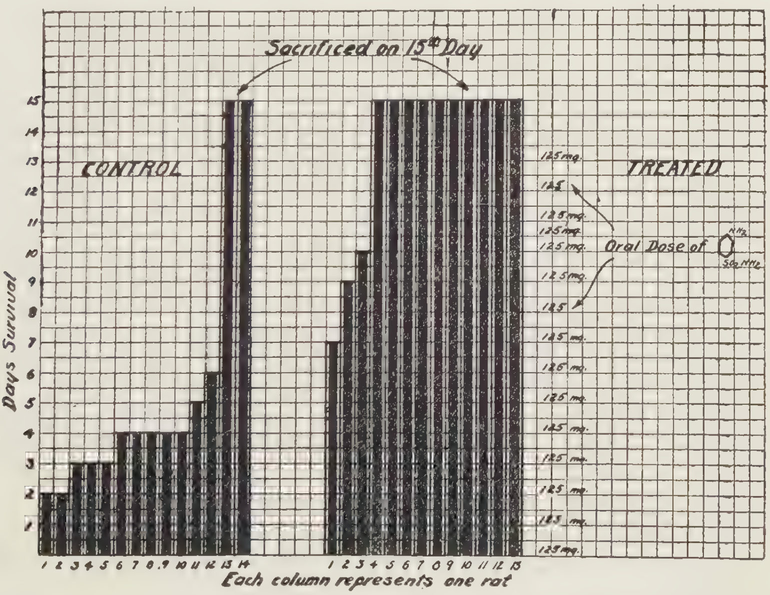

FIG. 1.

Graph showing survival time of rats infected intrabronchially with Type III pneumococci and treated with oral doses of *p*-aminobenzenesulfonamide.

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[1] Cooper, Frank B., Gross, Paul, and Mellon, R. R., PROC. SOC. EXP. BIOL. AND MED., in press.

[2] Rosenthal, S. M., *Public Health Reports*, 1937, **52**, 48.

[3] Gunn, F. D., and Nungester, W. J., *Arch. Path.*, 1936, **21**, 813.

[4] Nungester, W. J., and Jourdonais, L. F., *J. Bact.*, 1935, **29**, 34.

TABLE I.

	Acute Pneumonia	Chronic Pneumonia	Empyema	Peritonitis	Pleural Adhesions	Grossly Normal Lungs	Days Survival
Untreated Controls							
1	+++	—	+	—	—	—	2
2	++++	—	—	—	—	—	2
3	—	—	+	—	—	+	3
4	++++	—	—	—	—	—	3
5	+	—	+	—	—	—	3
6	+	—	+	+	—	—	4
7	+++	—	+	—	—	—	4
8	+++	—	+	+	—	—	4
9	++	—	+	—	—	—	4
10	++	—	+	—	—	—	4
11	++++	—	+	+	—	—	5
12	++++	—	—	—	—	—	6
13	—	++++	—	—	—	—	15*
14	—	—	—	—	—	+	15*
Treated with *p*-aminobenzenesulfonamide.							
1	—	—	+	—	—	+	7
2	++++	—	—	—	+	—	9
3	++++	—	—	—	—	—	10
4	—	—	—	—	—	+	15*
5	—	++++	—	—	—	—	15*
6	—	—	—	—	+	+	15*
7	—	—	—	—	—	+	15*
8	—	+	—	—	—	—	15*
9	—	—	—	—	—	+	15*
10	—	—	—	—	+	+	15*
11	—	—	—	—	—	+	15*
12	—	+	—	—	—	—	15*
13	—	+	—	—	+	—	15*

+ 25% involvement of one lung.
++ 50% " " " "
+++ 75% " " " "
++++ 100% " " " "
* Sacrificed on the 15th day.

apy would best approximate the results anticipated in human beings.

Twenty-seven rats (150-200 gm.) were infected with a dilute 24-hour broth culture of Type III pneumococci ("420" strain), suspended in mucin. Thirteen of these animals were given 125 mg. of *p*-aminobenzenesulfonamide* by mouth immediately, and subsequently as indicated in Fig. 1. The remaining 14 served as untreated controls. This latter group showed an 85.7% fatality, while only 23.1% died in the treated group. The autopsy results of these animals and of the survivors, which were sacrificed after 15 days, are given in Table I.

In view of these and previously obtained favorable results[1] sulfonamide therapy was tried on 9 out of 18 cases of Type III pneumococcus pneumonia at The Western Pennsylvania Hospital.[5]

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* Kindly supplied by The Winthrop Chemical Co., New York.

[5] To be published by others of this Institution.

Of the 9 untreated cases, 7 died (77.8%); whereas only 2 of the 9 treated cases died (22%).

Because many cases of human pneumonia come to the physician relatively late, further experiments are in progress to determine the efficacy of sulfonamide therapy in rat pneumonia which had been allowed to progress without treatment for various time periods.

Conclusions. *P*-aminobenzenesulfonamide therapy is effective in prolonging, and in many instances saving, the lives of rats infected intrabronchially with Type III pneumococci suspended in mucin.

9180

Filtration Studies on Pyrogenic Inulin.

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In a previous report,[1] it was shown that the pyrogen or fever-producing substance in tap water and a sample of aquarium water which, upon intravenous injection produces a fever in dogs, was removable by filtration of the water through a 200-second Zsigmondy membrane filter or a Seitz Serum No. 3 filter.* Likewise removable is the pyrogen in glucose and physiological saline solutions found to be pyrogenic in patients upon intravenous injection.[2] Because the former filter acts mainly by sieving and the latter by adsorption, it is concluded that the pyrogen in these fluids was of a particulate nature of an order of magnitude larger than 50 mμ as well as adsorbable.

In the course of their work on inulin clearance, Goldring and Smith[3] found that some samples of dahlia inulin injected intravenously gave a febrile reaction in man, characterized by chill, fever, headache, nausea, and lumbar pain. This symptomatology resembles closely that found in man following an intravenous injection of one of the pyrogenic fluids which we have investigated. One gram of a sample of inulin supplied us by Professor Smith was injected intravenously into a dog, resulting in fever, leucopenia, chill, nausea, and vomiting. This symptomatology likewise parallels that of dogs receiving pyrogenic infusion fluids intravenously.

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[1] Co Tui, McCloskey, Schrift, and Yates, PROC. SOC. EXP. BIOL. AND MED., 1936, **35**, 297.

* Due to error in labelling, these filters were called "Seitz EK filters" in our previous communication.[1]

[2] Co Tui, McCloskey, Schrift, and Yates, *J. Am. Med. Assn.*, in press.

[3] Goldring and Smith, PROC. SOC. EXP. BIOL. AND MED., 1936, **34**, 67.

In connection with the alleged toxicity of inulin, it may be mentioned that Nathan[4] used it for the production of anaphylatoxin. By incubating a suspension of inulin with guinea pig serum and then injecting the mixture into guinea pigs, he found restlessness, dyspnea, convulsions and agonized breathing resulting in death (anaphylaxis). When inulin in solution was used, however, there was no such manifestation. He concluded that its ability to initiate this toxicity depends upon the physical state of the inulin. DeKruif and German[5] found that inulin mixed in proportion 1:16 with normal rat serum produced a potent anaphylatoxin. Hanzlik and Karsner[6] found that inulin, without serum, injected intravenously into guinea pigs produced symptoms of anaphylactoid shock, namely, marked dyspnea, shallow respiration, cyanosis, and restlessness. When the animals were killed, autopsy revealed collapsed and congested lungs and cardiac dilatation. It will be recalled that in none of these studies was the temperature curve of the experimental animals taken into consideration.

In view of the similarity of the manifestations of pyrogenic inulin in both dog and man to those of pyrogenic infusion fluids, it seemed desirable to submit pyrogenic inulin to a similar filtration study.

As in our previous work, the types of filter used here were: Berkefeld filter "W", Seitz Serum No. 3 filter, and Zsigmondy filter of graded porosity (ultrafiltration). Two samples of inulin (684 and 684A), which Pfanstiehl and Company kindly furnished, were used. These were made in 10% solution. Where ultrafiltration was performed, the entire solution was filtered through but one membrane, thus limiting adsorption to that membrane only.

Table I shows that the pyrogen from both samples passes through a Berkefeld filter as well as a 42-second Zsigmondy ultrafilter membrane, but is held back by filtration through 2 pads of Seitz Serum No. 3 filter. But while a 200-second Zsigmondy membrane filter removed the pyrogen in 684, it did not hold back that in 684A, and a 300-second filter had to be used. This would suggest that the pyrogen is of a different particulate size in the 2 samples. Since the pyrogen is held back by a 200-second Zsigmondy membrane filter in one

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[4] Nathan, *Z. f. Immunitätsf.*, 1914, **23**, 204.

[5] DeKruif, P. H., and German, W. M., *J. Infect. Dis.*, 1917, **20**, 833.

[6] Hanzlik and Karsner, *J. Pharm. and Exp. Therap.*, 1920, **14**, 449.

TABLE I.
Inulin.

Lot No.	Subject	Vol. Injected cc.	Filter Used	Final Concentration	Total Dose, Gm.	Temp. Changes	Blood Count Changes, WBC per cmm.
684	Dog	55.0	0	10.0	5.5	101.8-104.6	29,300- 4,900
	,,	235.0	Berkefeld W	9.8	23.03	101.8-105.2	29,650- 2,600
	,,	50.0	42-sec. Zsig.	9.9	4.95	101.4-104.2	16,500- 4,200
	,,	250.0	200 ,, ,,	9.8	24.5	101.6-102.2	11,500-10,600
	Man	2.5	0	10.0	.25	98.2-102.2	8,600- 4,950
	,,	250.0	200-sec. Zsig.	9.8	24.5	99.8- 99.8	9,600- 8,400
	,,	260.0	Seitz (twice)	9.7	25.22	98.8- 99.2	
684A	Dog	10.0	0	10.0	1.0	102.0-104.8	17,500- 4,300
	,,	15.0	Berkefeld W	9.8	1.47	102.0-104.6	18,200- 3,600
	,,	250.0	200-sec. Z.	9.7	24.25	102.2-103.4	17,700- 8,600
	,,	260.0	300 ,, ,,	9.6	24.95	101.6-101.8	21,200-20,800
	,,	240.0	Seitz (twice)	9.6	23.04	102.2-101.8	18,500-19,200
	Man	2.5	0	10.0	.25	99.2-103.4	7,200- 3,600
	,,	230.0	200-sec. Z.	9.8	22.54	99.4-101.8	10,200- 9,200
	,,	250.0	300 ,, ,,	9.7	24.25	99.4- 99.2	
	,,	250.0	Seitz (twice)	9.6	24.0	99.2- 98.8	10,200- 9,800

WBC = Leucocytes.

case and by a 300-second in the other, but not by a 42-second filter, it is concluded that the removal was accomplished by sieving rather than adsorption. On the other hand, the removal of the pyrogen by the Seitz Serum No. 3, because of the nature of the filter, is by adsorption. Smith and his associates[7] have devised a method of purifying crude, toxic inulin into a non-toxic one with charcoal, probably utilizing the known adsorbing power of the latter substance.

Since after the treatment with charcoal by the Smith method, and after filtration through membranes as well as through an adsorptive filter, inulin can be injected into man and dogs without any symptoms, it is clear that the results obtained by Hanzlik and Karsner in the guinea pig are not reproducible in the human being or in the dog. The question arises whether the symptoms elicited by Hanzlik and Karsner as well as by Nathan and by DeKruif and German were not due to an artefact instead of to inulin itself.

Summary and Conclusions. 1. The febrile reaction elicited by intravenous injections of some samples of inulin is similar to that following the intravenous administration of pyrogenic infusion fluids in both dog and man. 2. The pyrogen in the inulin as well as that in the pyrogenic infusion fluid is filterable through a Berkefeld filter, but held back by filtration through 2 Seitz Serum No. 3 filter pads and by Zsigmondy membrane filters of from 200 to 300 seconds. 3. The pyrogen in inulin, like that in pyrogenic infusion fluids, is therefore of a particulate nature of a large order of magnitude. It is likewise adsorbable.

9181

Phosphorus Metabolism. VII. Course of Phosphorus in Alimentary Tract of the Rat.

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The main object was to compare the concentrations of total P and its fractions in the stomach contents, small intestinal contents, large intestinal contents and colon, and feces, on a P-free diet, with the concentrations on a normal diet. This should indicate some changes taking place, particularly to what extent P compounds are excreted

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[7] Smith and associates. Personal communication.

TABLE I.

Phosphorus Content and Distribution in the Alimentary Canal Contents of Rats on a Normal Diet and on a P-free Diet.

	Phosphorus in Contents						
	Total	Lipid		Organic Acid-soluble		Inorganic	
	mg. %	mg. %	% of total P	mg. %	% of total P	mg. %	% of total P
Average for Normal Diet							
Stomach	77	7.3	10.9	41	51.1	19	25.4
Small intestine	148	10.2	7.1	17	14.4	117	77.5
Large ”	741	8.2	1.2	195	25.9	394	53.7
Average for P-free Diet							
Stomach	31	4.3	12.2	7.7	47.3	—	—
Small intestine	167	15.0	8.9	8.2	5.5	121	72.2
Large ”	164	9.4	6.2	22.2	14.6	87	52.6

TABLE II.

Content and Distribution of Phosphorus in Intestinal Mucosa of Rats on a Normal Diet, a P-free Diet, in Fasting, and on High-fat and Low-fat Meals.

Exp. No	Diet	Phosphorus								
		Total	Lipid		Organic acid-soluble		Inorganic		Residual or ''Protein P''	
		mg. %	mg. %	% of total P	mg. %	% of total P	mg. %	% of total P	mg. %	% of total P
1	Normal	391	42	10.6	61	15.5	158	40.3	131	33.6
2		349	49	14.0	53	15.3	146	41.8	101	28.1
3		374	47	12.4	66	17.5	150	40.1	112	30.0
4		344	45	13.1	45	13.1	129	37.4	125	36.3
5		371	30	8.1	52	13.9	183	49.4	106	28.5
6	24-hr. fast	394	44	11.3	73	18.7	129	32.8	147	37.3
7		351	44	12.4	59	17.2	125	35.1	124	34.4
8	High-fat meal after 24-hr. fast	336	46	13.7	62	18.8	121	35.8	107	32.2
9		368	59	15.9	96	27.5	120	32.5	93	24.1
10	Low-fat meal after 24-hr. fast	385	38	9.8	71	18.3	161	41.6	116	30.3
11		389	34	8.7	64	16.9	150	38.1	142	36.4
12	Phosphorus free	383	51	13.4	61	15.9	179	46.8	91	23.8

into the alimentary tract not only by the saliva, gastric juice, bile and pancreatic juice, but also by the intestinal wall.

The "P-free" diet consisted of the following:

Starch-agar (32-1)	69
Gelatin	9
Egg albumin	9
Butter	10
Cod liver oil	1
Salts ($CaCO_3$ 1, NaCl 1)	2

It was found to contain 50 mg. % total P, 2.5 mg. of which were in phospholipid form. There was only a trace of inorganic phosphate.

Groups of 3 rats each were kept on the diets for one, 2, and 3 weeks; they were then killed and the different contents named above were analyzed for total P, and lipid-, organic acid-soluble-, and inorganic-P. Table I summarizes some of the results.

When the maximum amount of P coming from the saliva, gastric juice, bile and pancreatic juice was calculated for each period it was found that there was still a considerable excess of P in the feces for each corresponding period, even though no reabsorption from the above secretions was assumed. This indicated that P compounds, particularly phospholipids and inorganic phosphates, were excreted into the lumen through the wall. This is in harmony with the recent work of Shapiro, Koster, Rittenberg and Schoenheimer,[1] which showed that fecal fat in man originated to a large extent by secretion into the intestinal lumen.

Later the intestinal mucosæ of other similar rat groups were analyzed for the same P compounds under a normal diet, a 24-hour fast, 6 hours after ingesting a high-fat meal, and 6 hours after ingesting a low-fat meal. Table II presents the results.

Conclusions. There is a considerable secretion of P compounds into the intestinal lumen through the wall. This holds not only for phospholipids but particularly also for inorganic phosphates.

The intestinal content does not show any decided change in the concentration of total P, organic acid-soluble- or inorganic-P, on the different diets or when fasting for 24 hours. The concentration of lipid P, however, is just definitely higher on the high-fat meal, and definitely lower on the low-fat meal.

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[1] Shapiro, A., Koster, H., Rittenberg, D. and Schoenheimer, R., *Am. J. Physiol.*, 1936, **117**, 525.

9182

Modifications of the Rous-Turner Solution for Preservation of Bird Erythrocytes.*

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A concentration of 0.7% NaCl has been found by one of us (M.R.I) to be satisfactory for keeping the erythrocytes of various species of birds for relatively short periods of time, one to 2 weeks. In the hope of lengthening the time of survival of these cells, and at the same time retaining their agglutinability, modifications of the Rous-Turner formula[1] for mammalian cells were attempted. Cells from a species of dove *(Streptopelia risoria)*, of pigeon *(Columba livia)*, and of the domestic chicken *(Gallus gallus)* were used in about a 5% suspension with varying strengths of solutions.

A dilution of the Rous-Turner formula (9.2 gm. NaCl, 0.05 gm. $NaHCO_3$, 0.1 gm. KCl, 0.1 gm. $CaCl_2$) approximately isotonic with 0.7% NaCl was made by adding 32 cc. of distilled water to 100 cc. of the solution. For the trials, 100 cc. of the solution was diluted by the addition of from 28 to 34 cc. of distilled water. Equal parts of dextrose (Bacto) solution were added, in a concentration of 5.4% dextrose as recommended by Rous,[1] and, also, 4.2% dextrose, which was an amount arbitrarily taken as being proportional to the original solution of saline suitable for the preservation of bird cells. Only sterile solutions were used. These were tested in duplicate with each of the 3 types of cells.

The dextro-saline solution readily supported bacterial growth, even at refrigerator temperatures, making it necessary to collect the blood under as sterile conditions as possible. A satisfactory procedure consisted in first sponging the skin area over the wing vein with tincture of iodine, then with alcohol. The blood, in quantity to approximate a 5% suspension, was added directly to the tubes (16 or 18 mm. dia.) containing the different solutions. The mere dilution of the blood prevented an appreciable amount of clotting. For bloods not obtained with the above precautions, the addition of merthiolate even in 1:1000 was found to be insufficiently bacteriostatic for preservation purposes.

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† Formerly Research Assistant in Genetics.

[1] Rous, P., and Turner, J. R., *J. Exp. Med.*, 1916, **23**, 219.

TABLE I.

Preservation of Erythrocytes from Different Species of Birds in Various solutions.

	Weeks	1	2	3	4	5	6	7	12
.7% NaCl	Chicken	++	+++	+++	++++	C			
	Pigeon	—	+++	+++	+++	C			
	Dove	+	++	++	+++	+++	++++	C	
Locke's Solution	Chicken	—	+	++	+++	C			
	Pigeon	—	+++	+++	+++	C			
	Dove	+	+++	+++	++++	++++	++++	C	
100 cc. Locke's Sol. + equal parts 5.4% dextrose	Chicken	—	—	—	—	—	—	—	+
	Pigeon	—	+++	+++	+++	C			
	Dove	—	±	±	+	++	++	++·+	C
.7% NaCl + equal parts 5.4% dextrose	Chicken	—	—	—	—	—	—	—	+
	Pigeon	—	+	++	++	+++	C		
	Dove	—	±	±	+	+	++	+++	++++
100 cc. Locke's Sol. + 30 cc. dist. H_2O + equal parts 4.2% dextrose	Chicken	—	—	—	—	—	—	—	±
	Pigeon	—	±	+	++	+++	+++	++++	
	Dove	—	—	—	—	±	+	++	
100 cc. Locke's Sol. + 32 cc. dist. H_2O + equal parts 5.4% dextrose	Chicken	—	—	—	—	—	—	—	±
	Pigeon	—	$\underline{\pm}$	+	+	+	+	++	
	Dove	—	—	—	—	+	+	++	
100 cc. Locke's Sol. + 32 cc. dist. H_2O + equal parts 4.2% dextrose	Chicken	—	—	—	—	—	—	—	±
	Pigeon	—	$\underline{\pm}$	±	+	+	+	++	
	Dove	—	—	—	—	±	+	++	
100 cc. Locke's Sol. + 34 cc. dist. H_2O + equal parts 5.4% dextrose	Chicken	—	—	—	—	—	—	—	—
	Pigeon	—	—	—	—	$\underline{\pm}$	±	+	
	Dove	—	—	$\underline{\pm}$	±	+	++	+++	

Symbols: —, no lysis; $\underline{\pm}$, very faint trace; ±, faint trace; +, trace; ++, +++, ++++, well defined lysis in increasing amounts, respectively; C, complete lysis.

The tubes were stored at 3-5°C., and were examined for lysis at intervals of 7 to 9 days, at which time the tubes were shaken to assure thorough mixing. Lysis was measured roughly by the intensity and amount of coloration of the supernatant fluid.

The findings of 2 independent trials are summarized in Table I. These tests, although not giving identical results, agreed so closely that they are grouped together. Only those solutions with which favorable *results* were obtained in preserving the cells are given in the table. The other solutions not included gave in general less successful preservation of the pigeon and dove cells, and to some extent of the chicken cells. The addition to these various solutions of ¼ or ½% gelatine was of no aid in preventing lysis.

It is readily apparent in the table that chicken cells are much more easily preserved in dextrose solutions than those of either pigeon or dove, both as to length of time and in the range of dilutions possible. Furthermore, there is some indication that, in many of the dilutions given, dove cells were preserved longer than pigeon cells. The exception, in the solution containing 34 cc. of distilled water plus 5.4% dextrose, was observed in each of the separate trials. All these cells, however, were fairly well preserved in any one of the following combinations: (a) 100 cc. of Locke's solution plus 32 or 34 cc. of distilled water, (b) to which was added equal parts of either 4.2% or 5.4% of dextrose solution.

The agglutinability of the suspended cells with specific antisera was tried at the 6th week in the first test, and at the 8th week in the second. In all cases, the stored cells agglutinated at the same end dilution of the antisera as did fresh cells of each species. Whether or not each of the many antigens present in these cells[2, 3] will be fully expressed in like manner after standing is yet to be determined.

9183 P

Hyperglycemia Due to Impaired Hepatic Glycogenesis.

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It has been recognized that there is a difference in many respects between the severe type of diabetes mellitus occurring in the child

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[2] Irwin, M. R., and Cole, L. J., *J. Exp. Zool.*, 1936, **73**, 85; 1936, **73**, 309.

[3] Todd, Chas., *Proc. Royal Soc. (B)*, 1930, **106**, 20; 1931, **107**, 197.

and the mild type of glycosuria found in obese, middle aged people. An explanation defining this difference on a metabolic basis has not been presented. The following experiments indicate that in obese, middle-aged patients with glycosuria there is no deficiency in the oxidation of glucose. There is, however, a disturbance in the mechanism which removes glucose from the blood stream and deposits it as glycogen.

Normal controls were fed a diet containing 200 gm. of carbohydrate, 80 gm. of protein and approximately maintenance calories for 3 days. They then entered the respiration chamber.[1] The next morning they were instructed to arise, void, and imbibe 100 gm. of glucose in solution. Immediately after the glucose had been ingested a continuous 4-hour period of indirect calorimetry by the open circuit method[1] was obtained. The subjects disposed of 40 to 50 gm. of the ingested glucose by oxidation during this 4-hour period. The urine contained no glucose. Since the blood sugar of normal individuals under these circumstances has returned to the fasting level in 3 hours or less, it is evident that 50 to 60 gm. of the ingested glucose was disposed of by the deposition of glycogen in the liver (and possibly to some extent in the muscles).

Using the same technic, 5 obese patients with glycosuria, who came to the clinic to be treated for diabetes mellitus were found to oxidize normal amounts of glucose. The urine contained from 4 to 17.5 gm. of glucose in the 4-hour period. The glucose tolerance curves of these patients performed a few days before the studies in the respiration chamber were definitely diabetic in type. A typical example follows: Fasting, 161 mg./100 cc.; 1 hour, 300 mg.; 2 hours, 202 mg.; 3 hours, 178 mg.; 4 hours, 158 mg. All showed significant glycosuria during the test.

Eight patients of this type, all having glycosuria and diabetic types of glucose tolerance curves were placed upon submaintenance diets. No attempt was made to control the glycosuria other than the somewhat restricted carbohydrate necessitated by the limitation of calories. Glucose tolerance curves during the period of weight reduction exhibited a gradual return to the normal which was attained in all cases when a normal weight was reached. At this weight all are aglycosuric on normal diets and show no disturbance in carbohydrate metabolism by any test.

Since these patients when obese were able to dispose of a normal amount of glucose by oxidation but nevertheless became hyperglycemic when given 100 gm. of glucose, it is clear that they were unable to lay down glucose as glycogen at the normal rate. This delay accounts for the excess of glucose in the blood and the consequent glycosuria. Surgical removal of 50% of the dog's liver causes a similar delay in clearing the blood of extra glucose.[2]

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[1] Newburgh, L. H., Johnston, M. W., Wiley, F. H., Sheldon, J. M., and Murrill, W. A., *J. Nutrition*, 1937, **13**, 193.

Therefore, it appears to be true that hyperglycemia and diabetic glucose tolerance curves simulating milder forms of true diabetes mellitus may be produced by disturbances within the liver which are associated with obesity. Since the term diabetes mellitus implies a diminished capacity for oxidizing glucose, such patients as those described above do not belong under this heading even though they conform to the diagnostic criteria for diabetes as ordinarily interpreted. Their abnormal utilization of carbohydrate was corrected by simple reduction of weight to normal.

9184

Chemospecific Flocculation of Sterols by Anti-Sterol-Sera.

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Some years ago, one of us reported experiments concerning the antigenic qualities of cholesterol and derivatives of cholesterol, using the method of complement-fixation.[1, 2] Flocculation tests, which were not published, confirmed the results with complement-fixation. In the meantime, Hahn and Hazado[3] reported experiments on a flocculation of cholesterol by cholesterol antisera. Since the demonstration of a specific and sensitive flocculation of cholesterol by the serum of animals injected with cholesterol (and a protein *"schlepper"*) furnishes a new and valuable argument for the haptenic quality of sterols, our own data are now given.

The great sensitivity of sterol sols toward salts may be obviated by adding 2 volumes of water to the serum-dilutions. The specific

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[2] Coller, F. A., and Troost, F. L., *Ann. Surg.*, 1929, **90**, 781-793.

* c/o Lederle Laboratories, Inc., Pearl River, N. Y.

[1] Weil, A. J., and Besser, F., *Klin. Wschr.*, 1931, page 1941.

[2] Weil, A. J., and Besser, F., *Z. Immunforschg.*, 1932, **76**, 76.

[3] Hahn, F., and Hazado, W., *ibid.*, 1936, **88**, 16.

TABLE I.

Showing Specific Flocculation of a Sol of Cholesterol by Anticholesterol Immune Serum.

0.5 cc. H_2O and 0.25 cc. 1/6% sol of cholesterol are added to decreasing amounts of inactivated serum, diluted with saline to a volume of 0.25 cc.

Amount of serum, cc.	a Anticholesterol immune serum	b Serum of a normal rabbit
0.05	+++	—
0.03	+++	—
0.02	+++	—
0.01	++	—
0.006	+	—
0.004	+	—

Readings after 24 hrs. at room temperature.

Controls, containing either serum or cholesterol sol alone, showed no flocculation.

flocculation of sterol sols (prepared according to Keeser[4]) is very slow under such conditions, but is maximal in 24 hours at room temperature.

Table I gives an example of such an experiment.

We were able to produce flocculations with cholesterol, di-hydrocholesterol and oxy-cholesterol (Lifschütz) and the corresponding antisera. Data on the preparations used and the method of immunization are given in the papers of Weil and Besser. The qualitative and quantitative relations of specificity were the same as reported for the complement-fixation test, that is, there is some cross-reaction between cholesterol, di-hydrocholesterol, and oxycholesterol (Lifschütz), but the specific antigen showed always a much stronger flocculation and a higher titer than the others. None of our sera reacted with cholesterol dibromide, cholesterol acetate, cholesterol palmitate, or cholesterol oxide (Westfalen), nor was there any reaction of these sera with alcoholic extracts of beef heart. Sera of normal rabbits, including those of our rabbits before immunization, gave no reaction with any of these substances.

In some preliminary tests on the quantitative conditions of the cholesterol-anticholesterol flocculation we used twentyfold and fortyfold amounts of the mixtures of immune serum, distilled water, and cholesterol given in the table in order to determine the cholesterol content of the entire mixture, of the floccules, and of the supernatant in a total volume of 20 or 40 cc. These data, together with the content of cholesterol of the sols and the immune serum used, were in good agreement, so that our method may be used for the further quantitative study of antigen-antibody reactions of this kind.

On the basis of these experiments it may be stated that the amount of cholesterol flocculated by our method is proportional to the amount of antibody used. For instance, 10 cc. of a serum diluted 1:10 flocculated 11.4 mg. out of a sol containing 158 mg. % cholesterol, whereas the dilution 1:20 flocculated 5.5 mg., and the dilution 1:40, 2.6 mg. under the same conditions.

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[4] Keeser, *Biochem. Wschr.*, 1924, **154**, 321.

9185

Tests for the Blood-C. N. S. Barrier in Experimental Poliomyelitis.*

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It is well known that the mechanism regulating exchange between the blood and the central nervous system and spinal fluid is disturbed in all types of meningitis, allowing both normal blood constituents and foreign substances to pass into the spinal fluid (reviewed by Katzenellenbogen[1]). While a "meningeal stage" has been described in poliomyelitis, the origin of the cells in the subarachnoid spaces appears to be from the perivascular spaces of the medullary substance. This communication will give the results of several tests of the blood central nervous system barrier in experimental poliomyelitis. Such findings have a bearing on specific treatment, since a gross barrier defect would allow free passage of neutralizing substances from blood into the medullary substance and spinal fluid.

Flexner and Amoss[2] while showing that the virus did not regularly pass from the blood to the nervous system, suggested that a defect in the barrier might be of importance in the natural pathogenesis of this disease in man. The failure to find virus-neutralizing substances in the spinal fluid of man during convalescence,[3] although these substances are present in the blood of a large number of human convalescents even during and prior to paralysis,[4] could be explained by an intact barrier. In testing the barrier in experimental poliomyelitis with several types of antibodies, Shaughnessy, Grubb and Harmon[5] showed that none of these substances, except diphtheria antitoxin, passed from the blood into either the spinal cord or the spinal fluid. In the clinic, Flatau[6] found the barrier impermeable to acid fuchsin. The value of this latter observation is not great as the stage of the disease was not stated.

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* Supported by a grant from the President's Birthday Ball Commission for Infantile Paralysis Research.

[1] Katzenellenbogen, S., *The Cerebrospinal Fluid and Its Relation to the Blood*, Baltimore, The Johns Hopkins Press, 1935.

[2] Flexner, S., and Amoss, H. L., *J. Exp. Med.*, 1917, **25**, 525.

[3] Flexner, S., and Amoss, H. L., *J. Exp. Med.*, 1917, **25**, 499.

[4] Harmon, P. H., Harkins, H. N., Fahey, J. J., and Wasbotten, P. M., Proc. Soc. Exp. Biol. and Med., 1936, **34**, 585.

We have utilized 4 materials in tests of barrier permeability: 2 diffusible substances, acid fuchsin[6, 7] and nitrate[8] ion and two colloidal dyes, Congo red[9] and trypan blue.[10] Tests have been done by each method in both the preparalytic and the paralytic stage. The dyes were administered intravenously, acid fuchsin as 5 cc. of 5% solution, Congo red as 10 cc. of 1% solution and trypan blue as 5 to 8 cc. of 1% solution. Sodium nitrate was given subcutaneously as 2 cc. of 5% solution. Cistern punctures were performed respectively after 1½ hours, (fuchsin) 8 to 14 hours (Congo red) 8 to 16 hours (trypan blue) and 4½ to 5 hours (sodium nitrate). Fuchsin color was intensified by acid alcohol,[6, 7] sodium nitrate was estimated upon a rough quantitative basis by diphenylamine,[8] and Congo red was brought out as blue by dilute acid, while trypan blue was identified by its color alone. In each instance unused monkeys *(Macaca mulatta)* were tested as normal controls while similar animals to which normal horse serum had been given by way of the cisterna magna to produce an aseptic meningitis served as a second series of controls. After the dyes were given, examination of the blood demonstrated an intense coloration of the blood serum at the time of the cistern puncture. Before the test was again applied in the subsequent paralytic stage, a preliminary cistern puncture was performed to show that no dye had penetrated into the spinal fluid in the interim. If gross or microscopic blood was observed in any fluid, that specimen was discarded. Monkeys of the rhesus variety *(Macaca mulatta)* were used throughout. In several cases the animals were sacrificed and coloration of the gray matter noted.

A summary of these data is given in Table I. In no instance was

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[5] Shaughnessy, H. J., Grubb, T. C., and Harmon, P. H., Proc. Thirty-eighth An. Meeting Soc. Am. Bact., Indianapolis, Dec. 28-30, 1936, *J. Bact.*, 1937, Preprint, **32**, 47.

[6] Flatau, E., *Rév. Neurol.*, 1926, **1**, 5.

[7] Nador-Nikitis, I., *Zentralbl. f. Haut. u. Geschlechtskr.*, 1926, **20**, 478.

[8] Mestrezat, W., *Le liquide céphalo-rachidien normal et pathologique*, A. Maloine, Paris, 1912.

[9] Stern, L., Kassil, G. N., and Lockschina, E. S., *Compt. rend. Soc. de biol.*, 1928, **99**, 448.

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TABLE I.

Summary of Tests of the Blood-CNS Barrier in Experimental Poliomyelitis.

Substance Used as Test of Barrier	Poliomyelitic Monkeys				Normal Controls		Controls with Sterile Horse Serum Meningitis	
	Infected with							
	Virus from Man	PMV Strain	Total No.	No. with Test Substance in Spinal Fluid	Total No.	No. with Test Substance in Spinal Fluid	Total No.	No. with Test Substance in Spinal Fluid
Acid Fuchsin	5	1	6	0	6	0	2	2
Congo Red	3	6	9	0	5	0	7	6
Trypan Blue	1	5	6	0	4	0	6	0
Sodium Nitrate	6	2	8	3*	4	0*	3	2*

*Signifies concentration of sodium nitrate greater than 10 mg. per L. Three poliomyelitic monkeys showed a concentration of sodium nitrate in the spinal fluid of 20 mg. per L., while 2 of 3 animals having sterile horse serum meningitis had a concentration of 30 and 40 mg. per L., respectively.

dye found in the cerebro-spinal fluid in either the preparalytic or paralytic stage of acute poliomyelitis or in normal animals, whereas passage was noted in several instances in animals having sterile meningitis. Congo red, acid fuchsin and nitrate ion passed more readily than trypan blue. Indeed, passage into the cerebrospinal fluid by nitrate ion was demonstrated late in the paralytic stage. Examination of spinal cords at the intervals stated previously revealed coloration of the gray matter in preparalytic and paralytic poliomyelitis. This coloration was most intense with Congo red and acid fuchsin. No coloration was observed in normal monkeys.

Discussion and Conclusions. These tests demonstrate that the poliomyelitic monkey does not differ profoundly from the normal animal in regard to permeability of the blood-CNS barrier to colloids introduced into the blood. Most important of all, the increased permeability of the vessels of the meninges and choroid plexus seen in meningitis is not observed in the former disease. The coloration of the gray matter demonstrates an increased permeability of the small blood vessels of this region in poliomyelitis as contrasted to the normal animal. Indeed, Faber,[11] who recently has visualized by vital coloration, with trypan blue, the early lesions of experimental poliomyelitis induced by nasal inoculation of virus has been able to trace progressive vascularity through higher to lower levels of the central nervous system. This author concludes that increased vascularity precedes cellular infiltration in the reaction of the nervous system to this virus.

This data from experimental poliomyelitis suggests the application of these and other tests, that have already been standardized, for use in man to aid in clinical differentiation of encephalitis and poliomyelitis on the one hand from tuberculous meningitis and lymphocytic choriomeningitis on the other.

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